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Viper Plague Project

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14. ABSTRACT
The term Viper Plague was coined for a lethal disease state seen in snakes belonging to a private herpetological collection infested with ticks (Kiel *et al*, 2006). Viper Plague presented signs and symptoms in the reptiles that were characteristically nearly identical to the Erhlichia disease Heartwater, also known as Cowdriosis, a highly lethal livestock disease (a USDA non-overlap Select Agent pathogen, exotic). It was revealed that some of the snakes in the group were imported from Ghana, West Africa, which is a major source of Heartwater. The collection, which included vipers, cobras, bullsnakes, and rattlesnakes, were infested with the reptile specific *Aponomma latum* tick and the *Amblyomma maculatum* tick, an arthropod capable of transmitting Heartwater but has not been known to be associated with reptiles. After early investigation into infected samples, it was discovered through electron microscopy and tissue culture results that Viper Plague consisted of a rickettsial agent and a possible Type-D retrovirus. To date, type-D retroviruses have not been seen in a tick

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Table of Contents

List of Figures

1. Introduction

The term Viper Plague was coined for a lethal disease state seen in snakes belonging to a private herpetological collection infested with ticks (Kiel *et al*, 2006). Viper Plague presented signs and symptoms in the reptiles that were characteristically nearly identical to the Erhlichia disease Heartwater, also known as Cowdriosis, a highly lethal livestock disease (a USDA non-overlap Select Agent pathogen, exotic). It was revealed that some of the snakes in the group were imported from Ghana, West Africa, which is a major source of Heartwater. The collection, which included vipers, cobras, bullsnakes, and rattlesnakes, were infested with the reptile specific *Aponomma latum* tick and the *Amblyomma maculatum* tick, an arthropod capable of transmitting Heartwater but has not been known to be associated with reptiles.

After early investigation into infected samples, it was discovered through electron microscopy and tissue culture results that Viper Plague consisted of a rickettsial agent and a possible Type-D retrovirus. The rickettsiae are obligate intracellular gram-negative parasitic bacteria that are amorphous in size and shape. The ability of these organisms to use the tick as a vector or reservoir and to readily cause disease in humans (zoonosis) has already been established by other scientific investigators. Retroviruses infect animals over a wide spectrum of species, including humans, where they have been shown to cause varying malignant and non-malignant diseases. To date, type-D retroviruses have not been seen in a tick arthropod as either a natural vector or host reservoir. The following is a summary of the work, progress, and achievements accomplished for the Air Force Research Laboratory's Counterproliferation Branch's (RHPC) Viper Plague Project. This research, conducted between August 2007 and March 2009, dealt with the extended effort to genotype and characterize both the rickettsial-like and retrovirus components of Viper Plague.

2. Preliminary Research

The predominant amount of work performed early on with the Viper Plague project dealt with becoming familiar with the project altogether before pursuing the genotyping and characterization of the Viper Plague pathogens. That entailed becoming accustomed to the Viper Plague derived isolates in cell culture, the different cell lines that were maintained for experiments and sub-culturing of the isolates, as well as the previous research that had been performed by other RHPC scientists.

At the time the Viper Plague project was started, the cell lines maintained in the lab were Russell's Viper Spleen epithelial cells (VSW, ATCC #CCL-129), Russell's Viper Heart fibroblast cells (VH2, ATCC #CCL-140), the Bovine Pulmonary Artery endothelial cells (CPA 47, ATCC #CRL-1733), and Human Cervical epithelial cells (HeLa, ATCC #CCL-2). Research was conducted on each cell line to understand the best technique for the maintenance and cultivation of each cell line. A level of confidence was attained not only after the aforementioned research but from also working extensively with the cell lines.
To gain a better understanding of the cytopathic effects (CPE) caused by the components of Viper Plague, Viper Plague cell cultures, which had been established in various cell lines, were closely

observed and monitored. Minor experiments examining different variables dealing with the sub-culturing of the infection lines were also performed.

After working with the infected cell lines, it was noted that the bacterium produced a cell-fusion plaque area, while the retrovirus manifested in more of an aggregated cell foci plaque, with dark centers in each cell. Another in vitro characteristic of the infectious process discovered was that the supernatant from an infected cell culture was incapable of transferring infection once sub-cultured. Examples of the morphologically different cytopathic areas along with an image of uninfected VH2 cells as a comparison are provided in figures 1a-1c.

Figures 1a-c

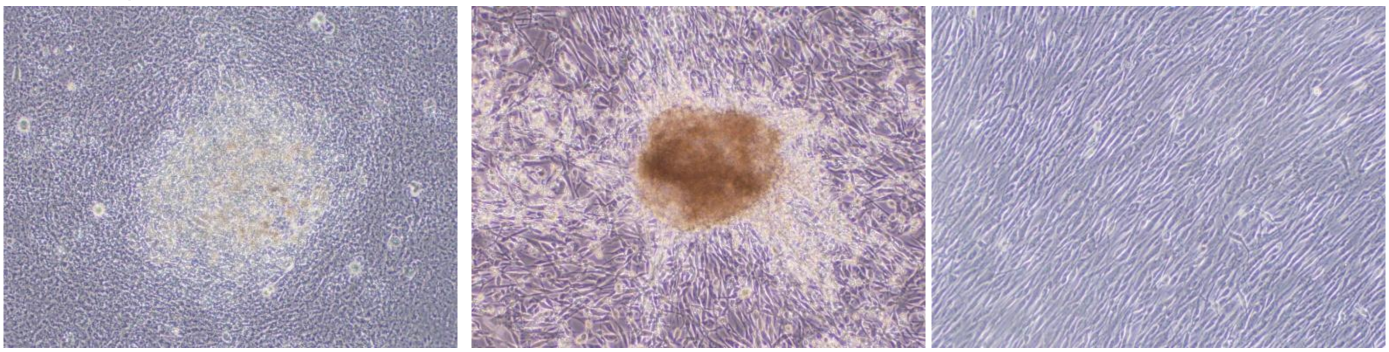


Fig. 1a. Characteristic plaque formation produced by the Viper Plague bacterium in VH2 cells, 10x.

Fig. 1b. Characteristic plaque formation produced by the Viper Plague retrovirus in VH2 cells, 10x.

Fig. 1c. Uninfected VH2 control cells, 10x.

Finally, an extensive literature review into rickettsial and retroviral pathogens was accomplished as well as a review of all previous Viper Plague research performed by RHPC. The goal was to ensure that the Viper Plague research plan would be the best and most efficient to characterize and identify the components of Viper Plague.

3. Western Diamond-backed Rattlesnake Infection

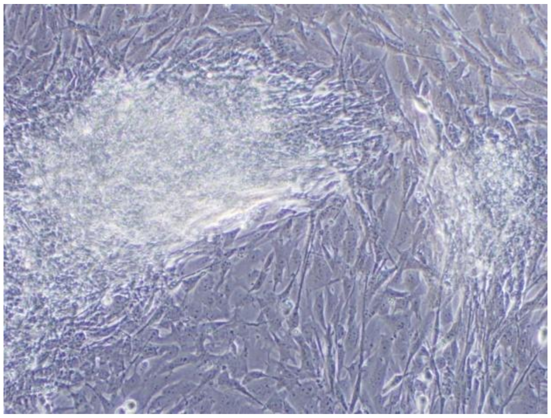
3.1 Establishment of Infection in Cell Culture

Infectious material from a Viper Plague infection maintained in vitro was used to inoculate a Western Diamond-backed Rattlesnake (*Crotalus atrox*). This was performed because this particular species of rattlesnake was present in the original herpetological collection, where Viper Plague was isolated. However, the Diamond-backed never acquired signs of disease as a result of Viper Plague.

The inoculated rattlesnake presented signs of gingival petechiae, mild head swelling, and extreme swelling and circulatory compromise to the tail, resulting in amputation at 87 days post-inoculation, but did not die of these complications. A blood sample was drawn from the snake and serum from the sample was used to inoculate VH2 cells. After confirming the in vitro results

through successive sub-culturing, it was determined that cytopathic activity could indeed be observed in each passage and that infection of the cell line was proven to have taken place. The following images (Figures 2a-b) are examples of the characteristic plaques associated with the Diamond-backed Rattlesnake infection line.

Figures 2a-b



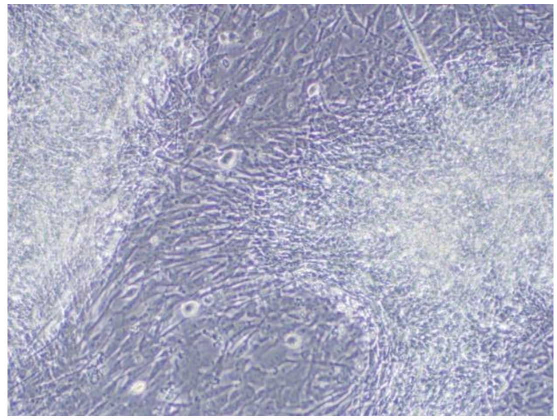
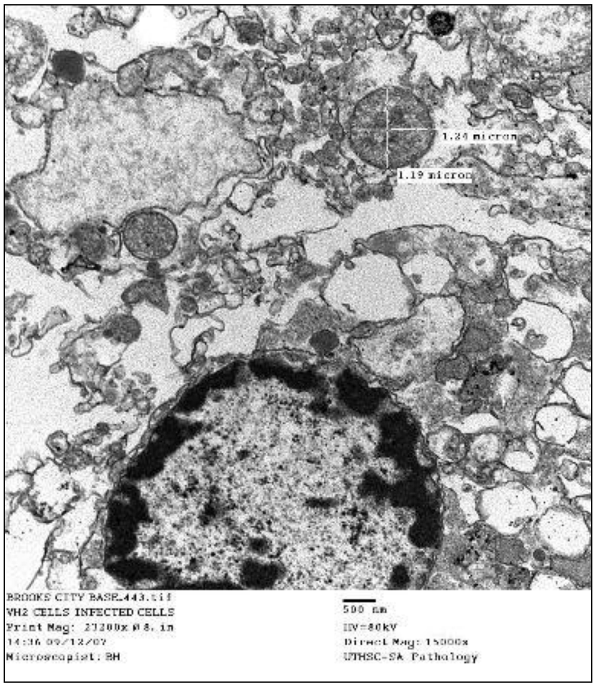


Figure 2a, 2b. VH2 cells infected with the Viper Plague rickettsial bacterium originally isolated from the Western Diamond-backed Rattlesnake; Pass 6, 10x.

3.2 Transmission Electron Microscopy (TEM) Preparations

For the purpose of gathering visual evidence that a rickettsial-like organism had been established in the VH2 cell line, preparations were made of the Diamond-backed infected VH2 cells along with uninfected VH2 control cells and delivered to the Electron Microscopy Laboratory at the University of Texas Health Science Center at San Antonio (UTHSCSA). The following images (Figures 3a-b) provide evidence that an intracellular bacterial pathogen is indeed present within the VH2 cell line. Remarkably, transmission electron microscopic scanning of the infected VH2 cells revealed the absence of the retrovirus from the Diamond-backedisolate. The conclusion was reached that either the immune system for this particular species of snake was able to eliminate the retrovirus infection or that the retroviral pathogen may have become latent within the genome of the rattlesnake because of an unsuitable host environment.

Figures 3a-b



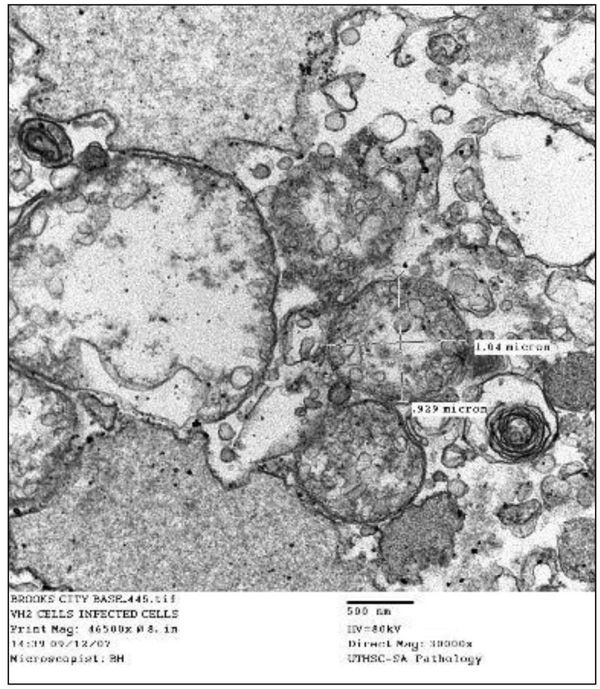


Figure 3a, 3b. TEM images of the sub-cultured Diamond-backed rattlesnake infection in VH2 cells. Structures considered being the Viper Plague bacterium are marked with the sizing cross-bars.

4. Febrile Agglutination (Weil-Felix) Test

4.1 Background

As a means to characterize the rickettsial organism of Viper Plague, the commercially available Proteus OX19 antigen (BD diagnostics, catalog #240782) and the Proteus polyvalent antiserum (BD diagnostics, catalog #240940) were incorporated with the intent of performing febrile agglutination tests. Proteus febrile antigens, molecular elements from the Proteus bacterium that induce fever and an antibody response, are used to diagnostically test for the presence of rickettsial antibodies. In the literature, cross-reactivity has been documented to occur between Proteus antigens and rickettsial antibodies, known as Weil-Felix reactions. It was proposed that the Proteus OX19 antigen and polyvalent antiserum could be used to indirectly test for the presence of a rickettsial organism.

For the testing of cell cultures infected with Viper Plague, the Proteus antigen and antiserum from BD diagnostics were utilized in a slightly different approach than the one used to test human sera. In following standard protocol, the Proteus antigen is applied to a small amount of sera and checked for agglutination while the Proteus antiserum is used only in combination with the antigen to serve as a positive control. Since the infection is maintained in vitro (i.e. in cell culture), the antiserum was applied to small amounts of infected cell culture to see if the Proteus antibodies

would cross-react with the rickettsial bacteria to form agglutinated particles. The Proteus antigen was used only in conjunction with the Proteus antiserum to serve as the positive control.

4.2 Testing of Bullsnake Cell Culture Samples

VH2 cells, inoculated with Viper Plague originally derived from an infected Bullsnake from the private herpetological collection, were screened for the presence of rickettsial antigens by utilization of the Proteus polyvalent antiserum. Two separate experiments were conducted where an attempt was made to release the rickettsial like organism (RLO) from the host cell through freeze thaw cycles at -80 °C alone and freeze thaw cycles at -80 °C plus sonication, respectively. Preparations were then treated with various amounts of Proteus antiserum and observed for signs of agglutination while the Proteus antigen with antiserum served as a positive control. Antiserum applied to a preparation of uninfected VH2 cells was included as the negative control.

No agglutination could be seen in the test samples or the negative control while the positive control exhibited agglutination. Even though the febrile agglutination testing of the Bullsnake infected samples failed to show signs of a rickettsial infection, the explanation may be that the intracellular bacteria were not fully released from the host cell to freely interact with the Proteus antibodies. Because of this possibility, febrile agglutination testing of Viper Plague derived infections strongly deserves further research.

4.3 Testing of Diamond-backed Rattlesnake Serum

Due to the results from the TEM images, a small amount of available Diamond-backed rattlesnake serum was tested with the febrile agglutination test components. For the first test sample, 50µL of snake serum was combined with 35µL of Proteus antigen while the second sample consisted of 50µL of snake serum treated with 50µL of Proteus antiserum. Agglutination could be seen in the test sample consisting of the rattlesnake serum plus antigen, while the serum plus antiserum test sample did not have any signs of agglutination. These results indicate that antibodies against rickettsial organisms were present in the serum while the presence of an actual rickettsial organism could not be detected even though cytopathic activity was established in cell culture.

5. Rat Snake Study

A rat snake was used as a test subject to prove the possibility of an immunosuppression link between the Viper Plague components and at the same time, prove Koch's postulates. The hypothesis was that both the bacterial and viral components of Viper Plague were necessary <u>and had to exist simultaneously within a host as a co-infection for disease manifestation</u>. Compromising the immune system of the host by either the bacterial or viral component initially would then allow the other to complete opportunistic killing of the host, the mechanics of which are not entirely understood.

The test specimen was inoculated first with the Viper Plague rickettsial component from the sub-cultured cells originating from the Diamond-backed rattlesnake, and then inoculated 48 hours later

with an infection line comprised solely of the retroviral virions. While there was some discoloration and swelling, there were no significant signs of disease. Lipopolysaccharide (LPS), an endotoxin from the outer membrane of gram negative bacteria, was then injected into the host snake two weeks later in hopes of eliciting a strong inflammatory immune response, which might ultimately promote disease. After signs of a mild infection and a return to normal, the rat snake was then inoculated four days later with Viper Plague infected cells originating from the Bullsnake, comprised of both the pathogenic bacteria and retrovirus. The test specimen again exhibited signs of a mild infection but no pathological signs of Viper Plague disease were seen.

A blood sample was collected three weeks later from the test specimen. VH2 cells were inoculated with only the serum at first, and when that did not produce signs of infectivity, a new VH2 monolayer was inoculated with the entire contents of the remaining blood sample. The inoculation of VH2 cells with the rat snake whole blood successfully produced plaque areas, characteristic of the rickettsial bacteria, and foci areas, a characteristic sign of the retrovirus. This leads one to conclude that the rat snake was successfully inoculated with Viper Plague and that infectious material, collected from the snake, was then able to infect a previously unchallenged cell line to reproduce the same effect in vitro, thereby proving Koch's postulates. The infectious culture will need to be furthered sub-cultured to be certain. The plaque formations and concentrated foci areas can be seen in the following images (Figures 4a-d):

<u>Figures 4a-d</u>

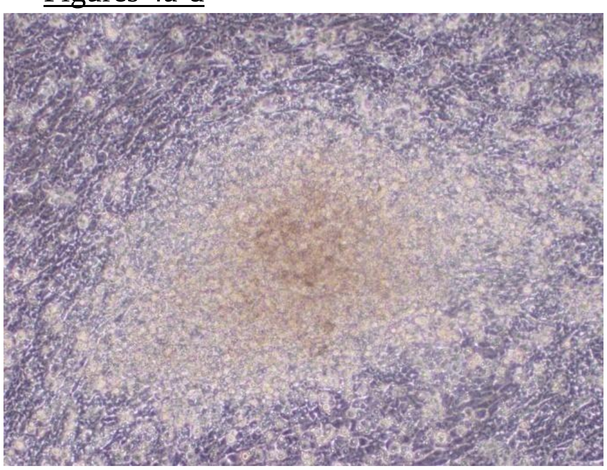


Fig. 4a. VH2 cells inoculated with the Rat snake whole blood sample; pass 0, post 1hr, 10x.

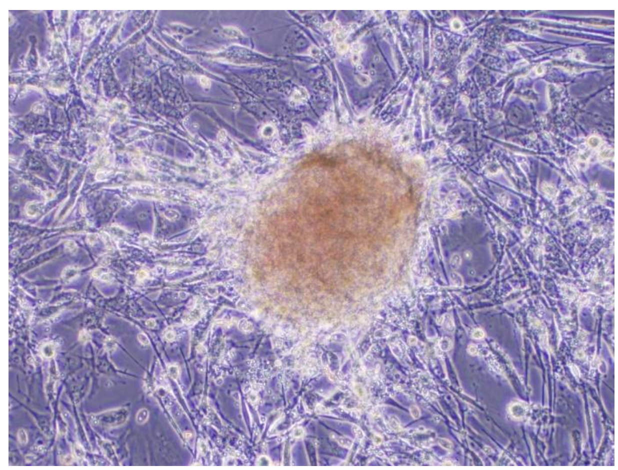


Fig. 4b. VH2 cells inoculated with the Rat snake whole blood sample; pass 0, post 9dys, 10x.

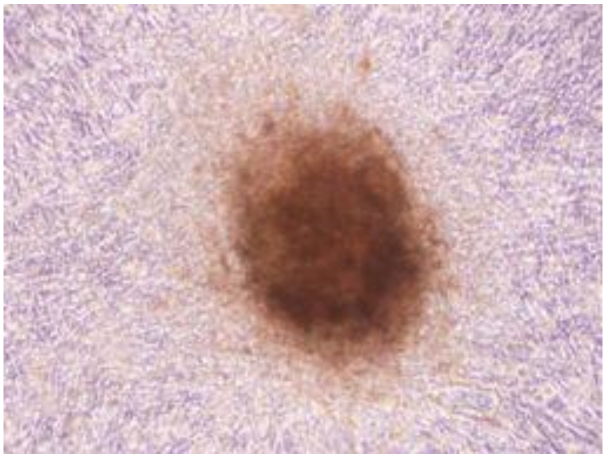


Fig. 4c. VH2 cells inoculated with the Rat snake whole blood sample; pass 1, post 20dys, 10x.

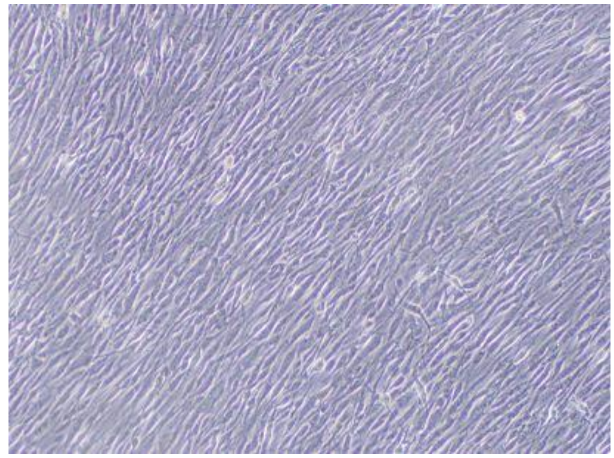


Fig. 4d. Uninfected VH2 control cells, 10x.

6. Antibiotic Susceptibility Study

An antibiotic susceptibility study was conducted in which VH2 cells were infected with the Bullsnake infection cell line, possessing both the rickettsial bacterium and the retrovirus, and then subsequently treated with either tetracycline or cycloheximide. Scientific literature has demonstrated that tetracycline is affective against the Rickettsia while cycloheximide has been shown to inhibit protein synthesis in most retroviruses. Infected host cells were either exposed to tetracycline hydrochloride (Sigma-Aldrich, catalog # T7660) at 3ng/ml, 30ng/ml, 300ng/ml, 3ug/ml, and 30ug/ml or Cycloheximide (Sigma-Aldrich, catalog #C7698) at 1ng/ml, 10ng/ml, 100ng/ml, 1ug/ml, and 10ug/ml in individual wells. The media, with the respective tetracycline concentrations, was changed every three to four days while the cycloheximide designated wells were exposed to the antibiotic, at the respective concentrations, for 18 hours and then replaced with fresh antibiotic-free media. The test was monitored over the span of a month.

For both antibiotic challenges, the highest concentration for each yielded the most noticeable effects. At post 28 days, the plaque areas associated with the Viper Plague bacterium were indeed inhibited by treatment with tetracycline. However, the CPE due to the retrovirus, cell aggregated foci areas, also seemed to be indirectly inhibited. The same correlation was noted in the wells treated with cycloheximide, the antibiotic known to inhibit protein synthesis, would indirectly affect the retrovirus. As the frequency of retroviral foci decreased with higher levels of cycloheximide treatment, the bacterial cytopathic activity also seemed to diminish to some degree. The results of this antibiotic susceptibility study, coupled with the pathophysiology recorded in the snakes, may suggest that a possible immunosuppression link could exist between the viral and the bacterial components of Viper Plague as a type of mutualistic relationship and is quite possibly necessary for disease manifestation in the host, through a mechanism which is not entirely understood as of yet.

Determination of Inter-species Infectivity for Viper Plague Derived Infection Lines

6.1 Bullsnake

While the rickettsial bacterium and the retrovirus originating from the Bullsnake had already been shown to infect various reptilian and bovine cell lines, an attempt was made to determine if the Viper Plague constituents were also capable of cross-infecting a human derived cell line. Infected VH2 cells were sub-cultured onto a monolayer of HeLa cells, a human derived cell line, and monitored for an extended period of time. After several weeks of observation and passage of the initial inoculation, CPE was observed; hence, successfully establishing the infectious process in the human cell line.

6.2 Western Diamond-backed Rattlesnake

After it had been demonstrated that the rickettsial organism and the retrovirus originating from the Bullsnake was capable of infecting a human derived cell line, an attempt was made to determine if the rickettsial agent isolated from the Western Diamond-backed rattlesnake was also capable of cross-infecting a human cell line. Since the isolate was, to this point, relatively newly established within the lab, this experiment was an effort to characterize further this particular isolate in cell culture. Infected VH2 cells were inoculated onto a fresh HeLa monolayer and monitored for an extended period of time. After several weeks of observation and passage of the initial inoculation twice, CPE was observed in the HeLa cells; hence, successfully establishing the infectious process in the human cell line.

7. Renografin Density Separation

An attempt was made to isolate the Viper Plague respective constituents using a Renografin density separation technique cited in Weiss et al., 1975. Using different concentrations of the Renografin reagent, a step gradient was formed that would separate the rickettsial and retrovirus based on the specific density of each. Work was performed using Viper Plague agents maintained in VH2 cells originally isolated from the Bullsnake and the Diamond-backed rattlesnake. Also included was an infected cell line consisting of only the retroviral organism isolated earlier in RHPC through a dialysis purification method. Infected VH2 cells, incubated between 3-4 weeks, were lysed in T-150s by treatment of the infected host cell layer with 1% Trypsin and incubation at 37 °C for 1 hour. After centrifugation at 2,000*g* for 15 minutes to pellet the host cell debris and a collection of the supernatant into a separate container, the pellet was then mechanically lysed using 18ga and 27ga needles. Samples were then centrifuged for a second time to re-pellet the host cell debris in an effort to collect the supernatant suspected of containing the rickettsial bacteria. All pooled supernatant was then ultra-centrifuged at 30,000*g* for 1 hour to pellet any rickettsial-like bodies. The concentrated pellet was then suspended in 2ml of 1x PBS pH 7.2 and purified using a Renografin 15% to 40% discontinuous gradient and ultra-centrifuged at 30,000*g* for 1 hour. The fraction of particular interest containing the bacteria, which separates around the 20% gradient as a visually distinct band, was processed through a second 15% to 40% Renografin discontinuous

gradient at 30,000*g* for another hour. The desired or fraction containing the organism fraction was then collected, washed with 10x volume of cold 1x Phoshphate Buffer Solution (PBS), and centrifuged again at 30,000*g* for 1 hour for purification. The pellet was subsequently re-suspended in 1ml of cold 1x PBS pH 7.2 and stored at 4 °C.

A sample of the re-suspended pellet was viewed under a light microscope with a Cytoviva light adapter for confirmation of bacteria purification and compared to another fraction from the same tube that separated at a different density. Small elementary light-refractive bodies could be seen in the fraction suspected of possessing the rickettsial bacteria while the other separation contained mainly crystalline formations and what could be described as host cell debris. To confirm that the bacteria was isolated using the density separation technique, 100µL was used to inoculate HeLa cells. No signs of infectivity could be seen in the HeLa cells after several weeks of monitoring.

VH2 cells were then inoculated, respectively, with the Renografin purified samples to determine if infectivity would occur, proving useful in validating if the Renografin purification was successful. At 28 days post-infection, positive signs of CPE could be observed in the VH2 monolayers. After five months, the Renografin purified inoculations continued to exhibit signs of cytopathicity. Please refer to the following images in figures 5a-d.

Figures 5a-d

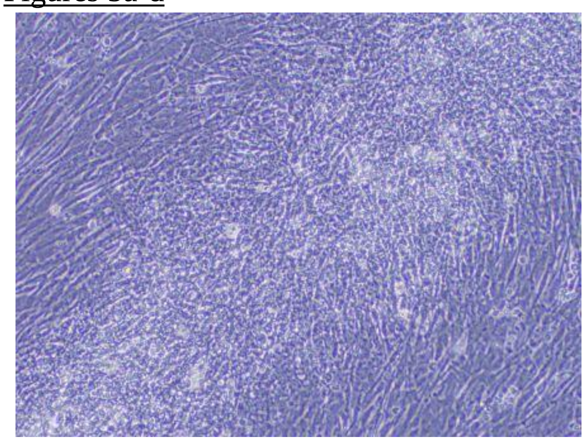


Figure 5a. VH2 cells inoculated with Renografin purified rickettsial agent from the Bullsnake isolate; pass 0, post 28dys, 10x.

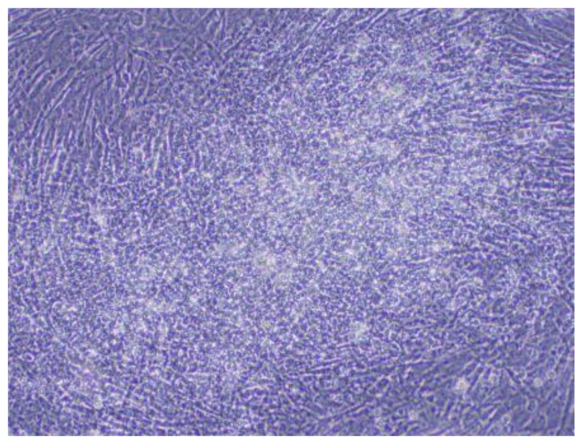


Figure 5b. VH2 cells inoculated with Renografin purified rickettsial agent from the Diamond-backed rattlesnake isolate; pass 0, post 28dys, 10x.

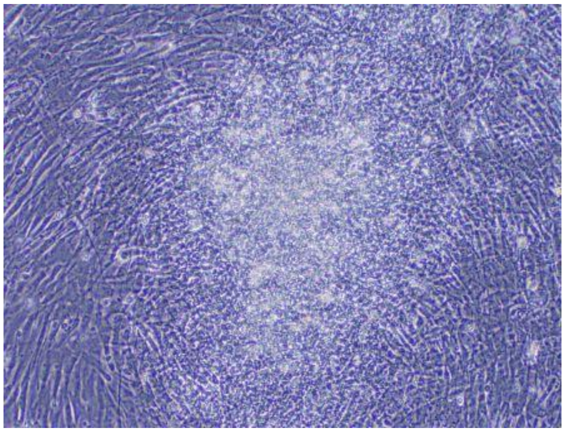


Figure 5c. VH2 cells inoculated with Renografin purified virions from sub-cultured viral infected VH2 cells; pass 0, post 28dys, 10x.

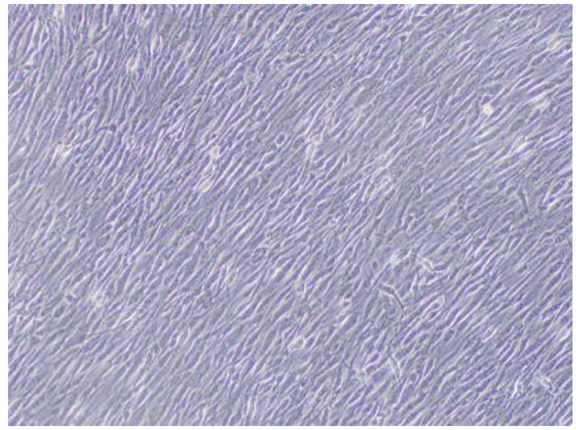


Figure 5d. Uninfected VH2 control cells; 10x.

Two other Renografin Density Separations were attempted, testing an increase in incubation time with the 1% Trypsin and processing the samples through only one discontinuous gradient instead of two, but both proved unsuccessful. On both occasions, histochemistry showed that the Viper Plague bacteria were not isolated. This may be explained by the likelihood that the bacteria were digested from an over-exposure to the high concentration of Trypsin during the extended incubation times. This observation proved influential in re-considering the role of the incubation with 1% Trypsin in all assays and procedures. After further testing and observing the results from these experiments, the decision was made to forego treatment of the Viper Plague isolates with 1% Trypsin because of the strong indication that the Trypsin was negatively affecting experimental assays due to the reagents strong digestive properties.

8. Histochemistry

8.1 Overview

For the purpose of visualizing the intra and extra-cellular rickettsial bacteria, the commercially available Diff-Quik staining kit from Dade-Behring (catalog number B4132-1A), which has been used in Ehrlichia and Rickettsia research publications to stain both intracellular parasites, was incorporated for the microbial identification of the rickettsial bacterium. Staining efforts began first on individualized cells, progressed to the staining of infected monolayers, and moved to the attempted staining of the Renografin purified organism. Significant progress was made applying the Diff-Quik kit in an effort to visualize the rickettsial bacterium. The following histochemistry involved using solely the bacterial pathogen originally isolated from the Diamond-backed rattlesnake, an isolate comprised only of the rickettsial organism.

8.2 Single Cell Infections

A protocol was developed for staining individual infected cells by first working on uninfected VH2 cells which would serve as a control in eventual experiments. After trypsinizing uninfected VH2 cells and heat fixing 20uL of the cell suspension onto microscope slides, the sample slides were stained using a slightly modified Diff-Quik staining procedure. The change in staining protocol was due to results from earlier testing. While the previous attempts to Diff-Quik stain uninfected VH2 stains produced cells that were too heavily stained at 100x magnification under the microscope, the slight modification to the staining protocol resulted in VH2 cells stained at an appropriate level where the organelles could then be well visualized and differentiated from the cytoplasm of the stained cell. Please see the images below (Figures 6a-b).

Figure 6a-b

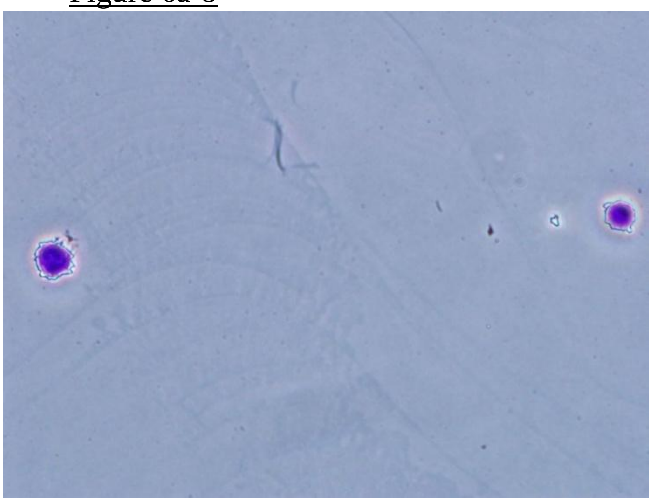


Figure 6a. Diff-Quik stained VH2 control cells, 40x

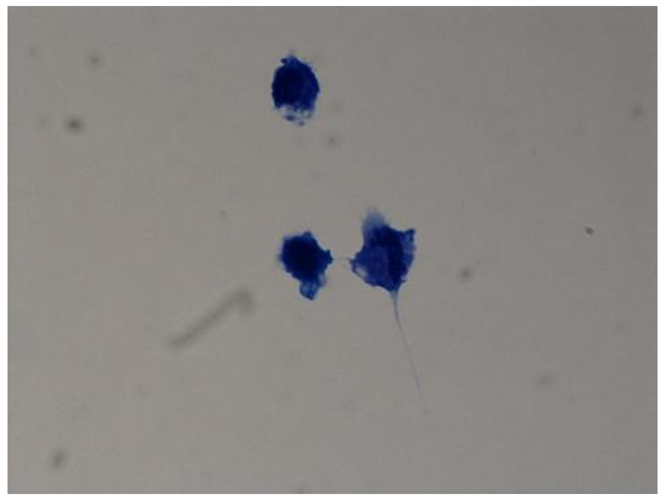


Figure 6b. Diff-Quik stained VH2 control cells, 100x

Once a staining protocol had been established for the non-infected host cells, efforts moved to staining VH2 cells infected with the rickettsial pathogen. After cell scraping infected cells from 6-well plates, a homogenous solution was created by gently aspirating and vortexing the infectious material. Twenty microliters of sample were placed onto glass slides and allowed to air dry. The fixed samples were then Diff-Quik stained using the slightly modified protocol provided by Diff-Quik. A control of uninfected VH2 cells to serve as a comparison against the infected VH2 cells was included and processed identically.

As can be seen from viewing figures 7a-d, there is a significant difference from the infected VH2 cells and the control. The control cells are stained a dark blue throughout, while the cytoplasm of the infected VH2 cells stained a reddish-pinkish hue and exhibited evidence of vacuolization. Another difference between the infected cells and the uninfected control cells was the presence of small bodies that were stained blue within the cytoplasm of the cells.

Figures 7a-d

Figure 7a. Diff-Quik stained uninfected VH2 control cells, 40x.

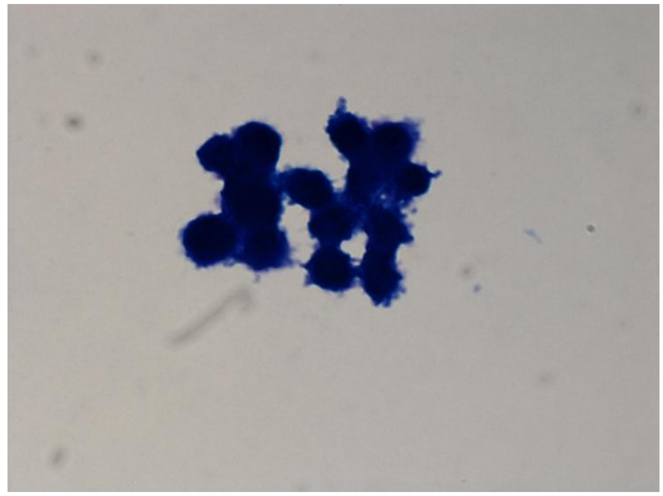


Figure 7b. Diff-Quik stained uninfected VH2 control cells, 100x.

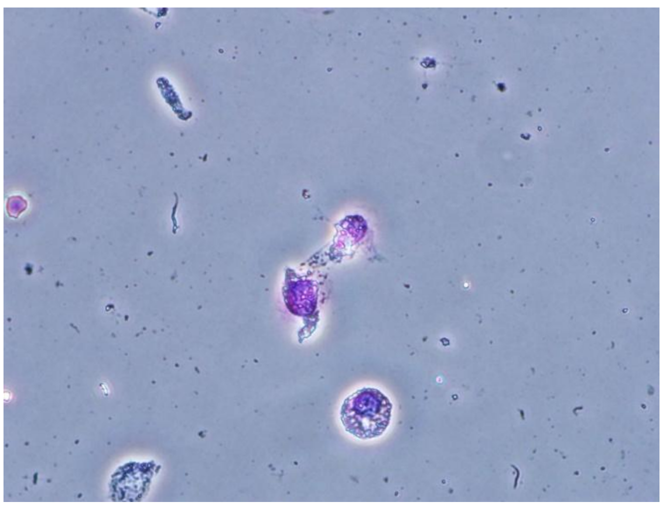


Figure 7c. Diff-Quik stained VH2 cells infected with the Viper Plague rickettsial organism, 40x.

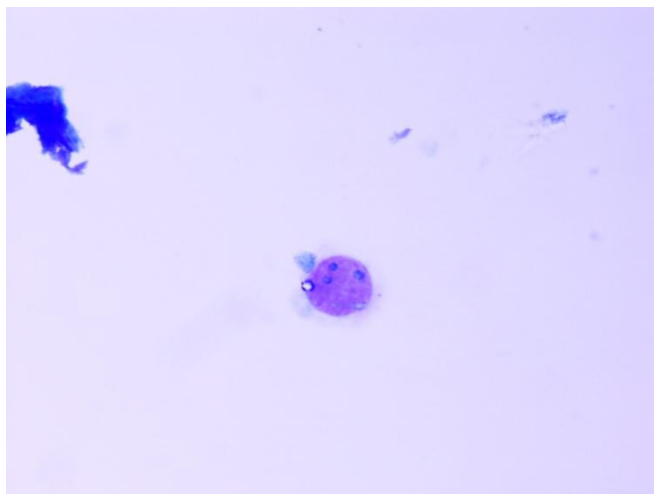


Figure 7d. Diff-Quik stained VH2 cells infected with the Viper Plague rickettsial organism, 100x.

8.3 Cell Culture Plate Format

Once individualized infected VH2 cells had been stained and visualized, the next task was to Diff-Quik stain a monolayer of infected VH2 cells still adhered to the well of a tissue culture plate. This is a staining application that has not been previously seen in the literature related to rickettsial research. After a simple protocol of washing the infected VH2 cells with PBS and using a modified Diff-Quik staining procedure, the wells were allowed to air dry before viewing under the microscope. A control of uninfected VH2 cells to serve as a comparison against the infected VH2 cells was included and processed identically. The following images (Figures 8a-c) present an

obvious difference from the control and the rickettsial infected samples. More so, the infected samples produced striking areas of stained plaques with a spectrum of colors between blue and red.

Figures 8a-c

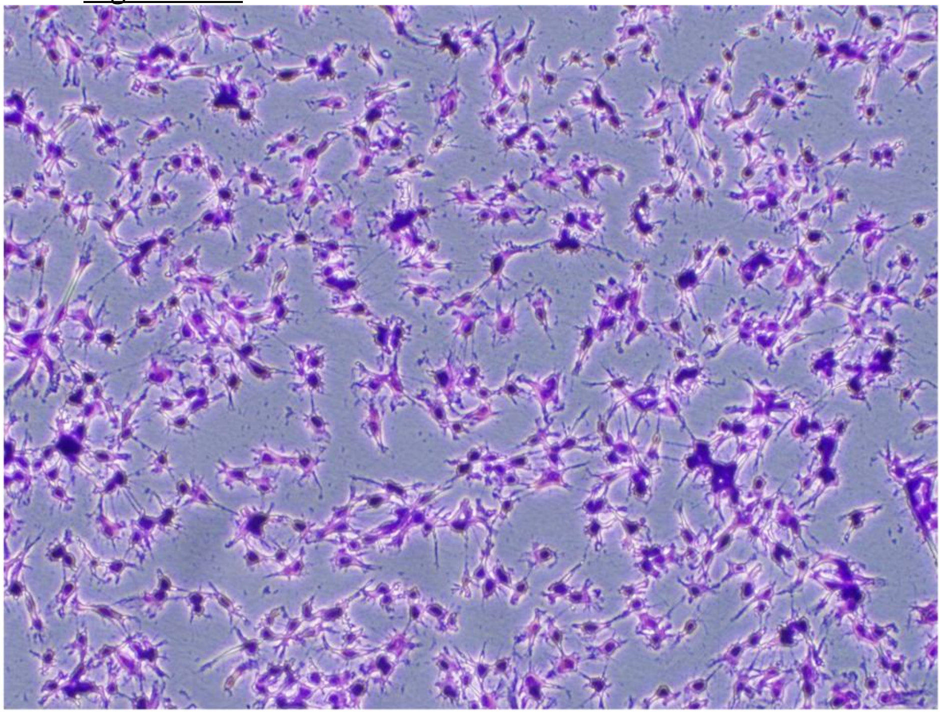


Fig. 8a. Diff-Quik stained monolayer of uninfected VH2 cells in a 6-well plate, 10x.

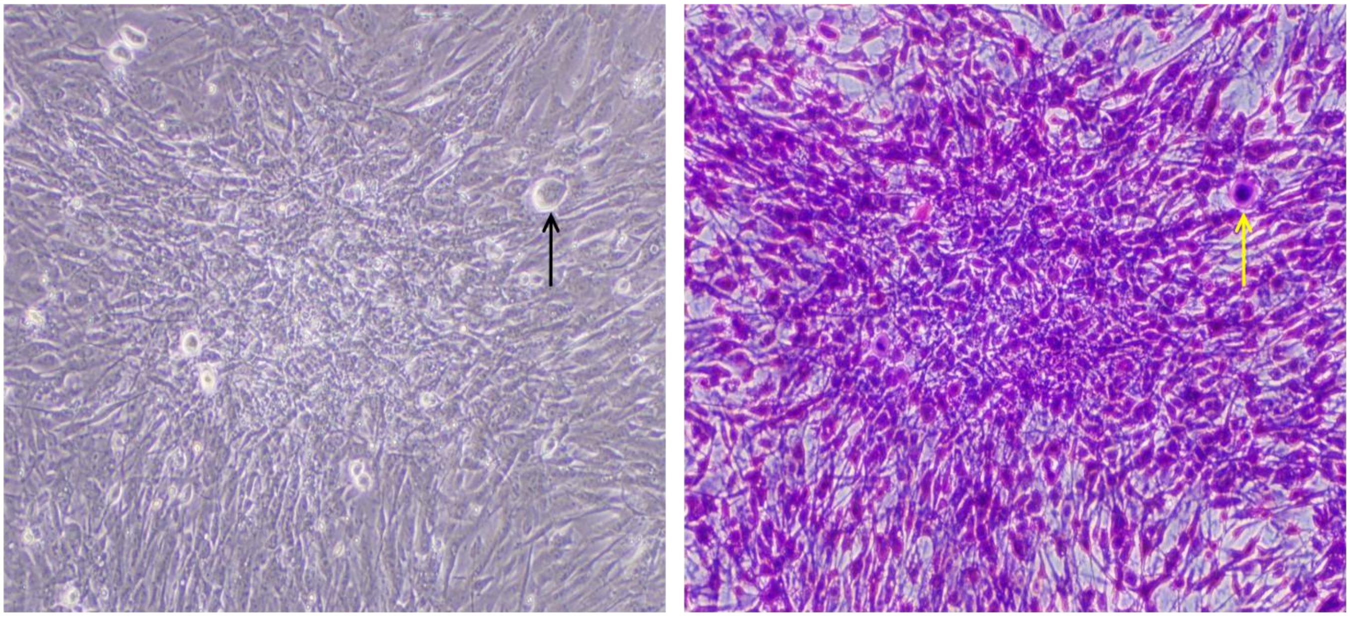


Fig. 8b. VH2 cells, in a 6-well plate, infected with the Viper Plague rickettsial organism maintained in the Western Diamond-backed rattlesnake infection line, pre- and post-staining, 10x.

Approved for Public Release, Public Affairs Case file no. 09-561, 22 December 2009; Brooks City-Base, Texas 78235.

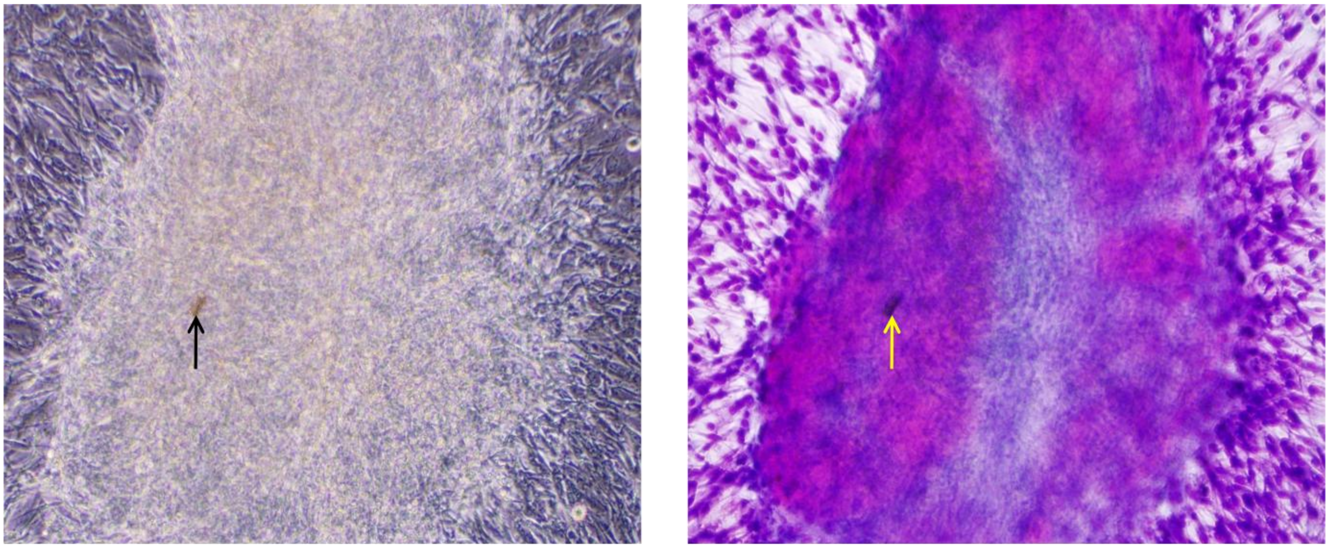


Fig. 8c. VH2 cells, in a 6-well plate, infected with the Viper Plague rickettsial organism maintained in the Western Diamond-backed rattlesnake infection line, pre- and post-staining, 10x.

From the above images, a definite difference in cell morphology does exist between cells infected with the Viper Plague rickettsial agent originating from the Diamond-backed rattlesnake and non-infected host cells.

8.4 Renografin Purified Rickettsial Organism

A Renografin Density Gradient Separation was performed on sub-cultured samples having the rickettsial organism and/or the retrovirus. In an attempt to microscopically visualize the rickettsial agent of Viper Plague, slide preparations were made of the stocked Renografin purifications. Due to the poor staining of rickettsiae with the gram staining technique, the commercially available Diff-Quik staining kit from Dade-Behring (catalog number #B4132-1A) was used to stain the fixed slide preparations. Rickettsiae are gram-negative organisms which have been described as possessing a blue color when stained using the Diff-Quik staining kit.

From the following images (Figures 9a-b), small individualized structures (highlighted with arrows) can be seen at 100x magnification and are stained with the characteristic blue color for gram negative bacteria. These small bodies possess two forms, bacillary (reticulated) and coccobacillary (dense-core), which is consistent with the pleomorphic nature of rickettsiae reported in scientific literature on rickettsia research. These images provide evidence that the Viper Plague rickettsial organism can be isolated in a pure culture using a novel separation technique based on the specific density of an organism. However, because of the sheer size of this pathogen, this process must be repeated and more scientific research must be performed to be sure before making the final determination that, undeniably, the bacterial component of Viper Plague can be isolated in the lab.

Figures 9a-9b

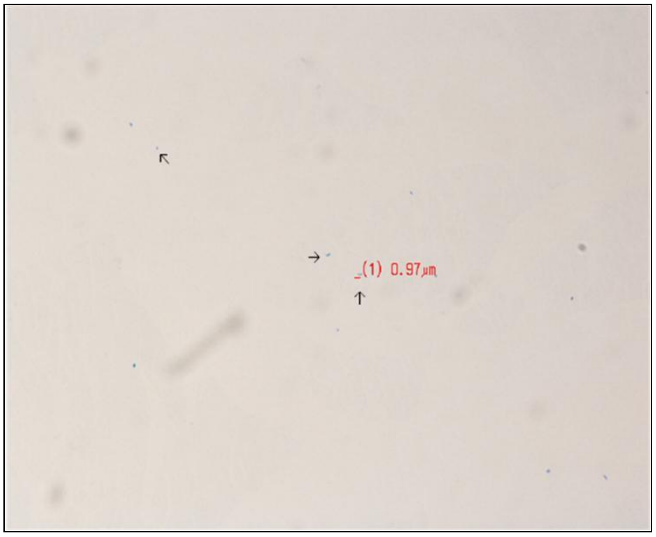


Figure 9a. Diff-Quik stained Renografin purified rickettsial-like organism (arrows) from the Western Diamond-backed rattlesnakeisolate, 100x.

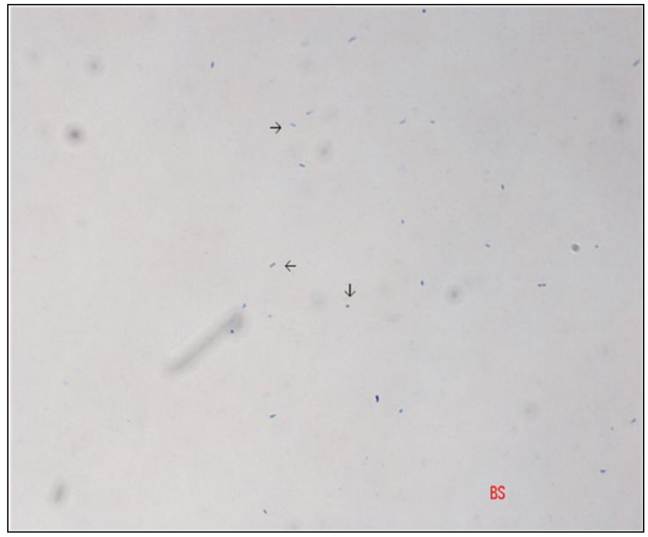


Figure 9b. Diff-Quik stained Renografin purified rickettsial-like organism (arrows) from the Bullsnake isolate, 100x.

9 Immunochemistry

The Proteus polyvalent antibody from BD Diagnostics febrile agglutination test was identified as a possible candidate for the development of a rapid field detection method of rickettsial infections. The antibody aids in serving as a diagnostic tool against rickettsial diseases by cross-reacting, through Weil-Felix reactions, with Spotted-Fever Group and Typhus Group rickettsiae. It was theorized that if the Proteus antibody was tagged with a fluorescent molecule the target rickettsial organism would be visually detectable once the conjugated antibody had bound, creating an antibody labeling method. The fluorescently-tagged antibody was created by conjugating the Proteus polyvalent antibody to the Alexa Fluor 488 fluorescent molecule (Invitrogen catalog #A-10235). Experiments were performed to test the specificity of the conjugated Proteus antibody under different parameters (Figures 10a-l), with the Viper Plague bacterium as the test organism of main interest. Compared to the controls, testing showed that the Proteus conjugated antibody was indeed specific for *Proteus vulgaris* while demonstrating no cross-reactivity when tested against other gram negative (*Pseudomonas aeruginosa* and *Vibrio parahaemolyticus*) and gram positive (*Staphylococcus aureus* and *Streptococcus pyogenes*) bacterial organisms. After demonstrating its usefulness, the conjugated Proteus antibody has been utilized in a wide variety of applications within the lab.

Figures 10a-l

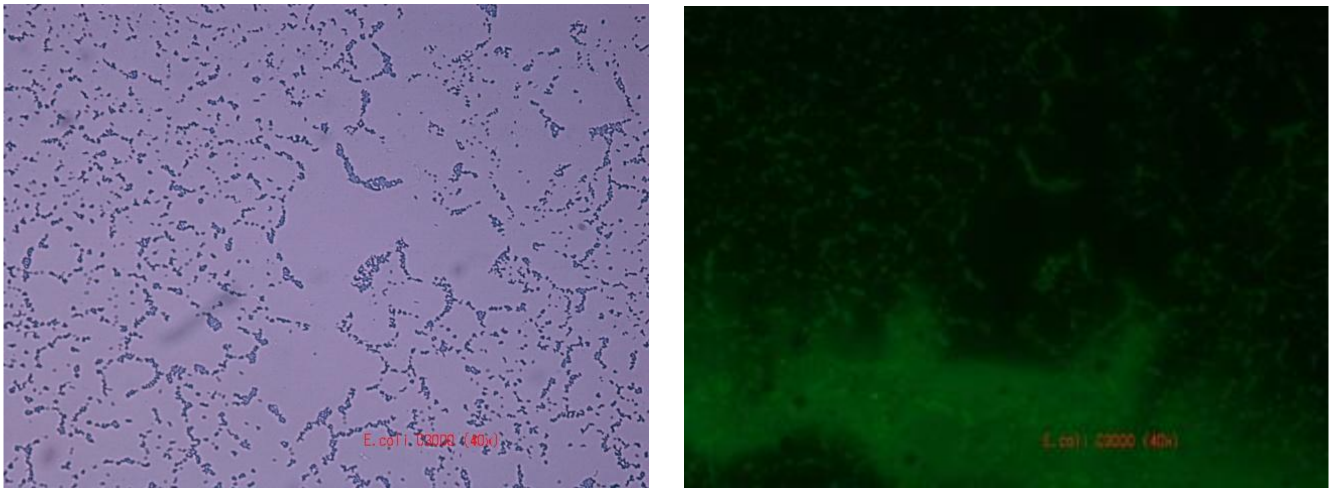


Figure 10a, 10b. E. coli strain C3000, negative control, labeled with the conjugated Proteus antibody, 40X.

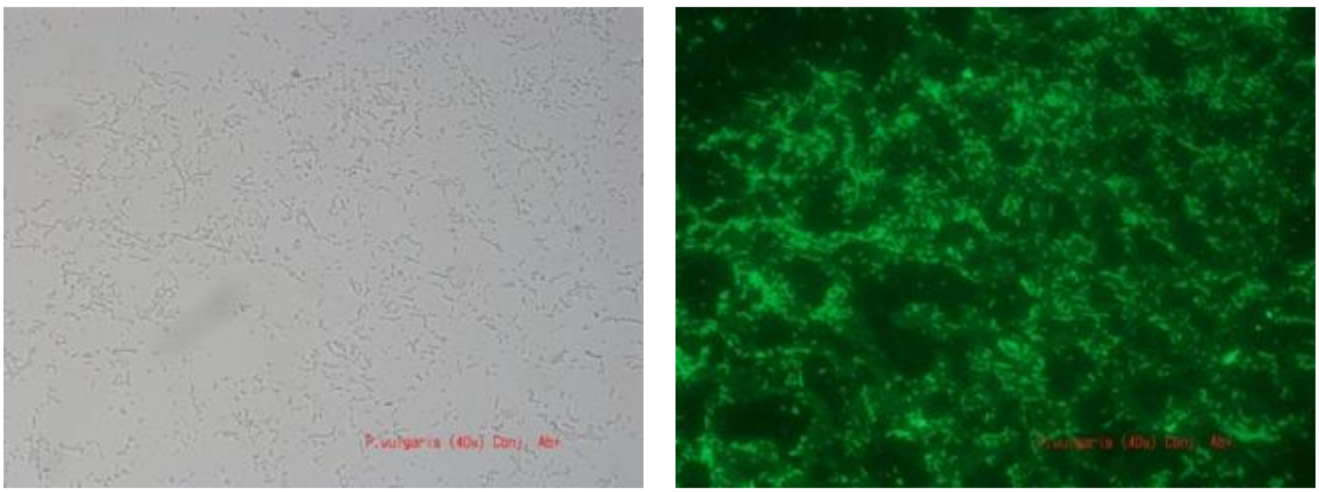


Figure 10c, 10d. *Proteus vulgaris* (gram negative), positive control, labeled with the conjugated Proteus antibody, 40x.

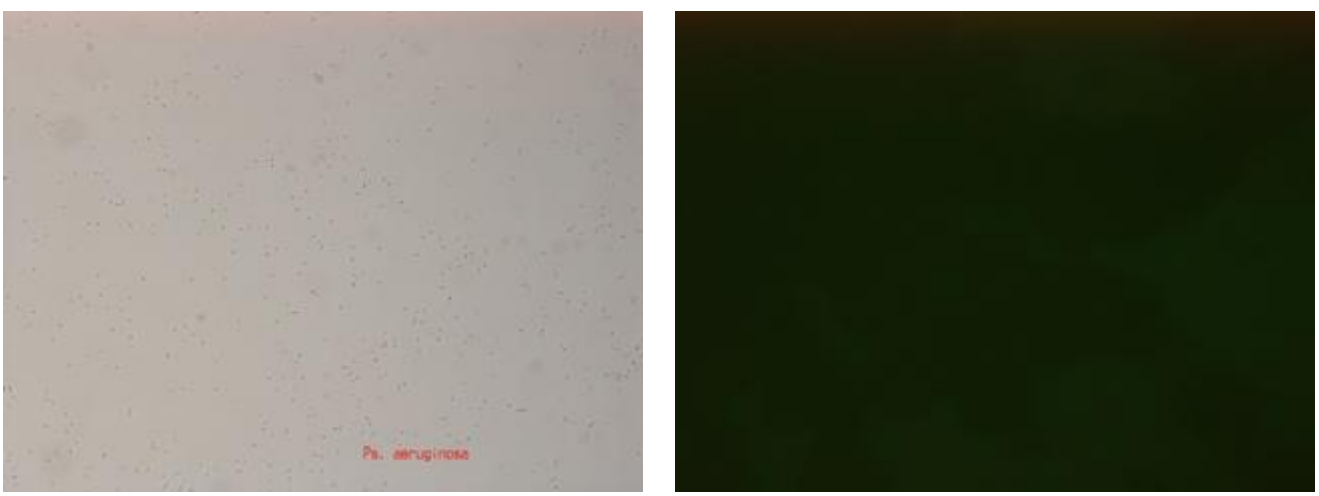


Figure 10e, 10f. *Pseudomonas aeruginosa* (gram negative), labeled with the conjugated Proteus antibody, 40x.

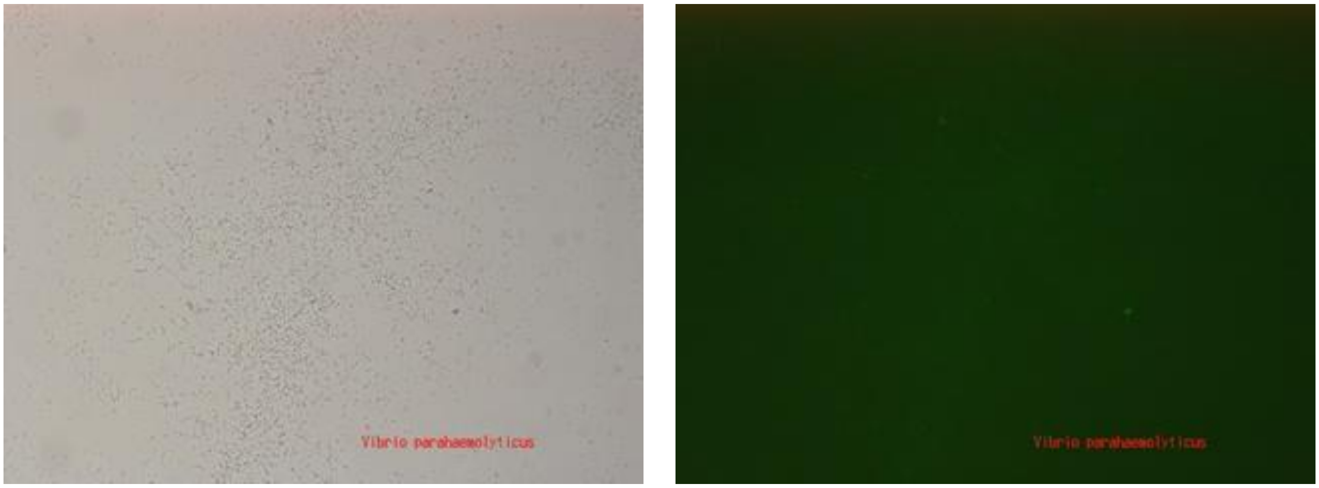


Figure 10g, 10h. ***Vibrio parahaemolyticus*** **(gram negative), labeled with the conjugated Proteus antibody, 40x.**

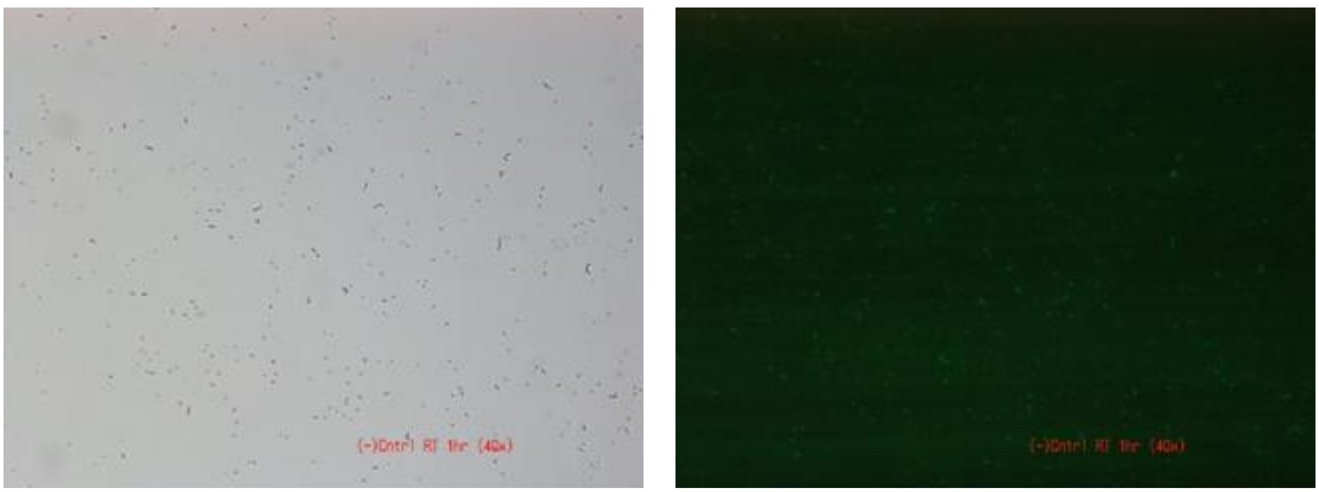


Figure 10i, 10j. ***Staphylococcus aureus*** **and** ***Streptococcus pyogenes*****, (gram positive), labeled with the conjugated Proteus antibody, 40x.**

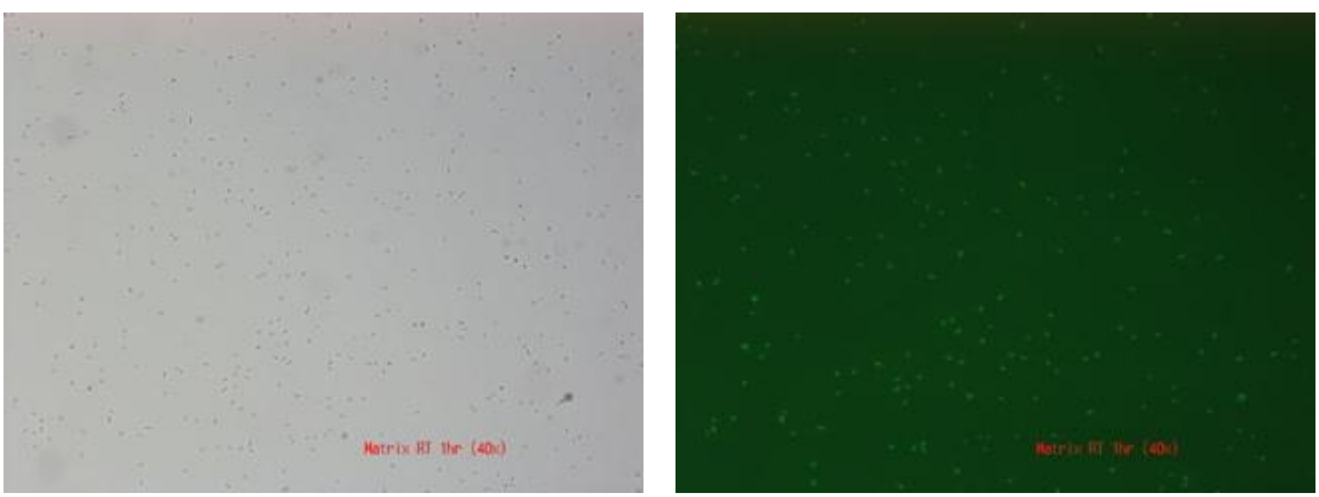


Figure 10k, 10l. Matrix test assessing the level of non-specific binding for the conjugated Proteus antibody against combined *P. vulgaris, S. aureus,* and *S. pyrogenes,* 40x.

10 Proteus OX19 Aptamer

10.1 Background

With the goal of developing a rapid collection method for use in the field as well as in the laboratory, a *Proteus vulgaris* OX19 aptamer was synthesized. Aptamers, or DNA capturing elements, are oligonucleotide sequences that have a high affinity to specific molecular targets. The Proteus OX19 aptamer was selected, using the Proteus OX19 antigen from BD Diagnostics as the template due to its cross-reactivity with the rickettsiae, namely the Spotted Fever Group and the Typhus Group rickettsiae.

10.2 Preliminary Testing

Testing was performed under various formats using the Proteus aptamer against the rickettsial organism to determine if this might be a possible candidate for a method of detection. Early testing of the Proteus aptamer consisted of conjugating the aptamer oligonucleotide to a fluorescent molecule in order to conduct labeling experiments similar to the antibody labeling technique. Results were ambiguous and inconclusive because of the high level of non-specific binding that occurred between the conjugated aptamer and host cell debris. The results from these early experiments were not an indication of the efficacy of the aptamer itself but, rather, that the testing format for the aptamer needed to be altered.

10.3 Proteus Aptamer-Magnetic Bead Construct

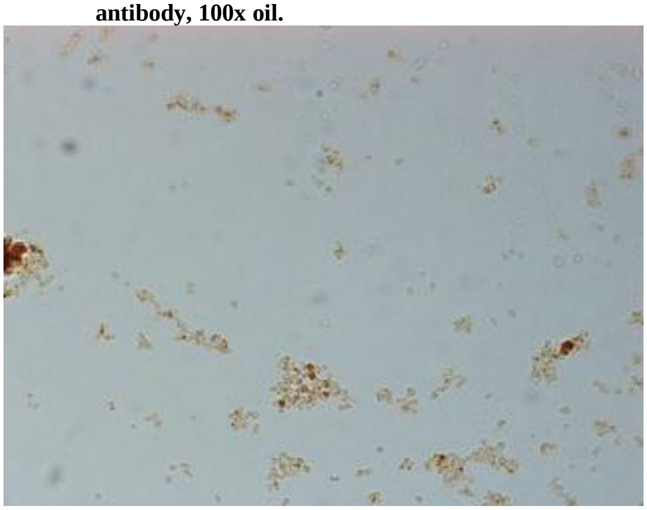
A different system to assess the binding efficacy of the *Proteus vulgaris* OX19 aptamer was proposed that involved coupling the Proteus aptamer to a magnetic bead as a means to select for rickettsiae from field samples. After first testing a 280nm iron-core magnetic bead, a 1.17µm polystyrene-core bead, coated with an iron magnetite layer plus an amine functional group layer, was finally selected for further experimentation. Initial results from the testing of the Proteus aptamer-magnetic bead (ProApt-Mag) system were very promising. By using the ProApt-Mag system to “pull out” the rickettsial organism from infected VH2 cells and subsequently inoculating a healthy monolayer of VH2 cells with the beads, it was ascertained that infection was transferred from one generation of VH2 host cells to the next. A pattern to the level of CPE was also observed in which infected VH2 samples treated with the ProApt-Mag system conferred a greater level of infectivity than samples treated with beads alone but less than purely sub-culturing the infection onto a new monolayer of VH2 cells. Controls revealed that that the ProApt-Mag system, as a complete system or as individually tested components (i.e. aptamer alone and bead alone), was not cytotoxic to the host cells.

10.4 Sandwich Assay

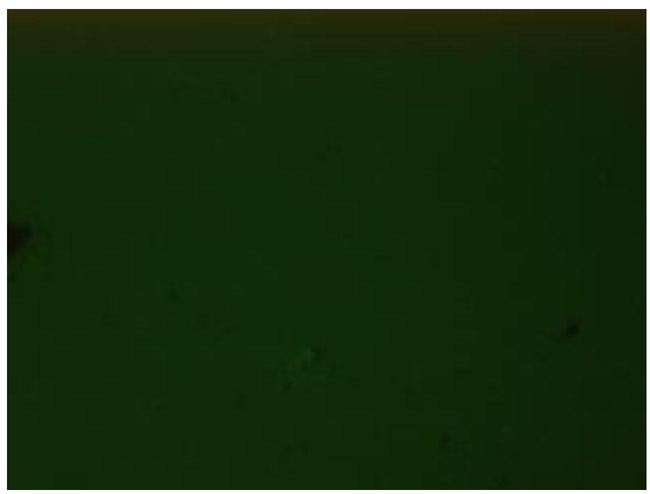
A sandwich assay was developed utilizing both the ProApt-Mag system and the conjugated fluorescent *Proteus vulgaris* OX19 antibody to visualize the rickettsial organism, bound to the aptamer-magnetic bead construct. The samples processed were from the ProApt-Mag bead experiments. To help in visualization, samples were Diff-Quik stained which allowed for viewing of the host cell in the microscope's bright field mode. Samples were labeled with the conjugate Proteus antibody using the antibody labeling protocol developed in-house for previous assays. In the following images, the infected samples treated with either the beads alone or the ProApt-Mag system possessed a high fluorescent signal while the other sample groups failed to emit, by comparison, a fluorescent signal in the acceptable range (Figures 11a-j).

Figures 11a-j

Figure 11a, 11b. Polystyrene 1.17µm magnetic beads only labeled with the conjugated fluorescent *Proteus vulgaris* OX19 antibody, 100x oil.



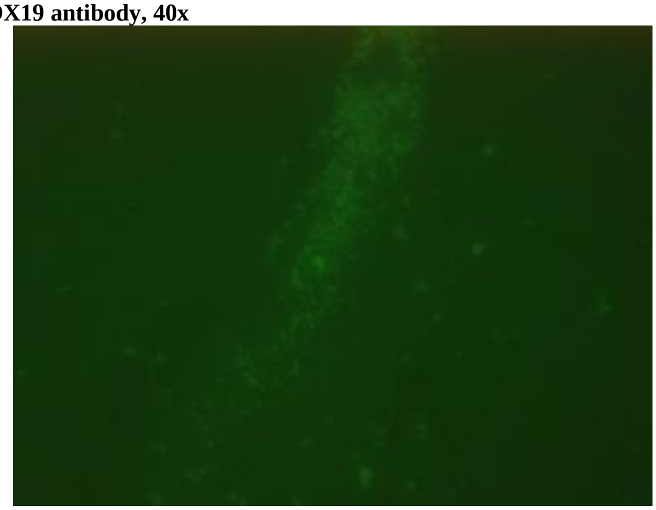
11a, bright-field image



11b, fluorescent image.

Figure 11c, 11d. Uninfected VH2 cells treated with only Polystyrene 1.17µm magnetic beads, Diff-Quik stained, and labeled with the conjugated fluorescent *Proteus vulgaris* OX19 antibody, 40x

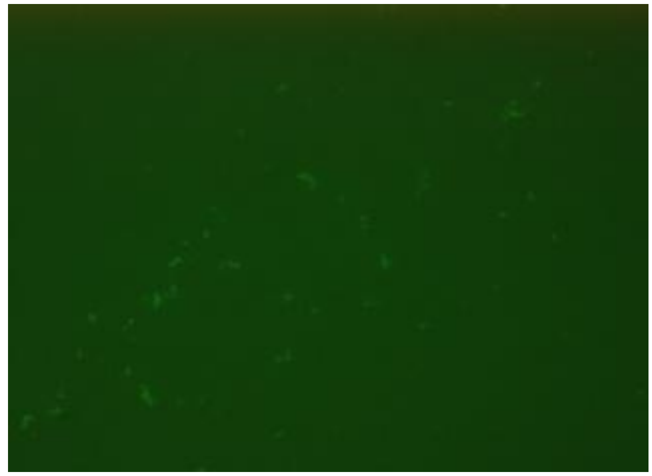
.11c, bright-field image



11d, fluorescent image.

Figure 11e, 11f. Uninfected VH2 cells treated with Proteus Apt-Mag complex, Diff-Quik stained, and labeled with the conjugated fluorescent *Proteus vulgaris* OX19 antibody, 40x.

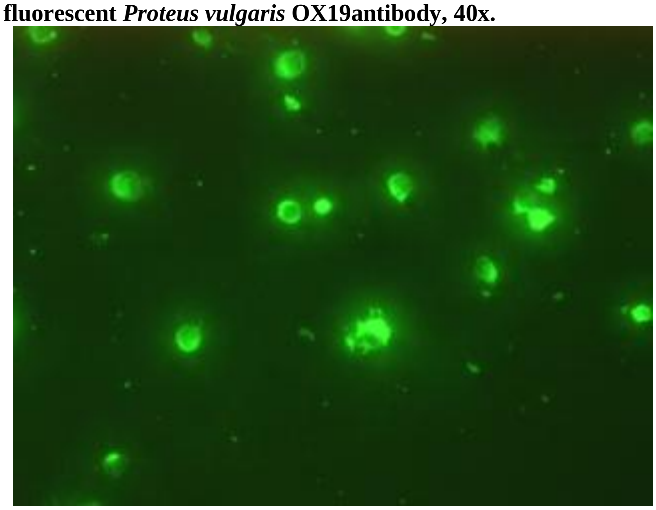
11e, bright-field image



11f, fluorescent image.

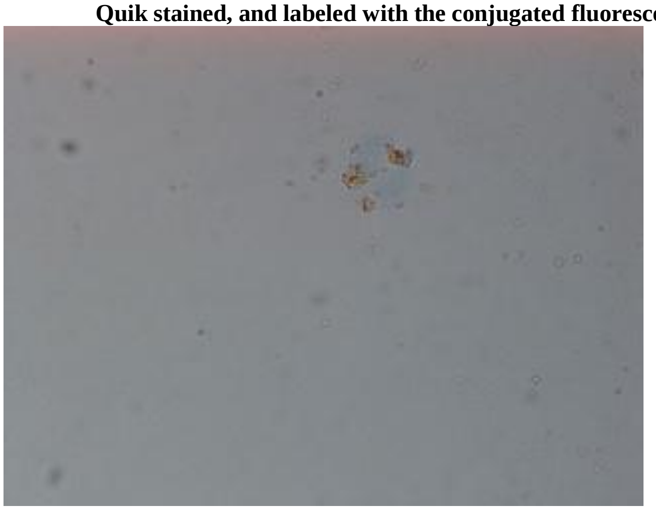
Figure 11g, 11h. VH2 cells infected with the Viper Plague bacterium and treated with only Polystyrene 1.17µm magnetic beads, Diff-Quik stained, and labeled with the conjugated fluorescent *Proteus vulgaris* OX19antibody, 40x.

11g, bright-field image

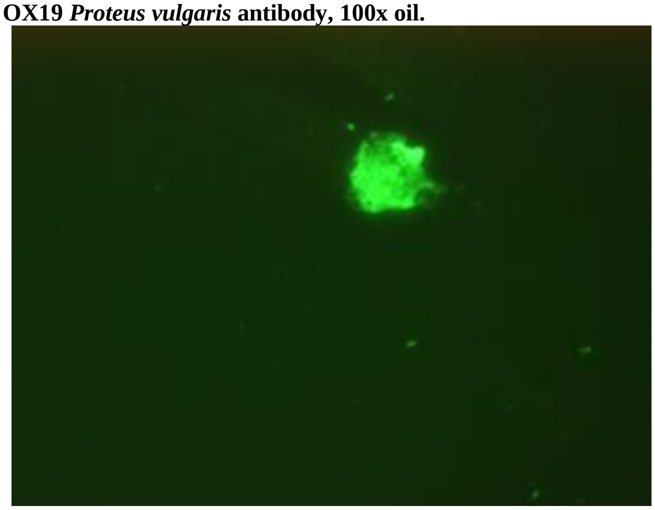


11h, fluorescent image.

Figure 11i, 11j. VH2 cells infected with the Viper Plague bacterium and treated with Proteus Apt-Mag complex, Diff-Quik stained, and labeled with the conjugated fluorescent OX19 *Proteus vulgaris* antibody, 100x oil.



11i, bright-field image



11j, fluorescent image.

10.5 Transmission Electron Microscopy Imaging

For the last experiment testing the *Proteus vulgaris* OX19 aptamer magnetic bead system, samples from the experiment were sent to the Electron Microscopy Laboratory at the University of Texas Health Science Center at San Antonio (UTHSCSA) for TEM imaging. The following TEM images provide evidence that the polystyrene magnetic beads were internalized by the host cell (Figure 12a). The layer of iron magnetite is represented by the light dark to dark shaped formations surrounding the area of the bead. In the second TEM image (Figure 12b), a distinct bacillary elongated form (indicated by the arrow) 0.2 - 0.4µm in length is seemingly attached to the bead by a microscopic bridge. Unfortunately, this structure was noticed only after the EM imaging session so there was no focus at the time of searching for more of these elongated bacillary bodies such as the one seen in Figure 12b.

Figure 12a-b

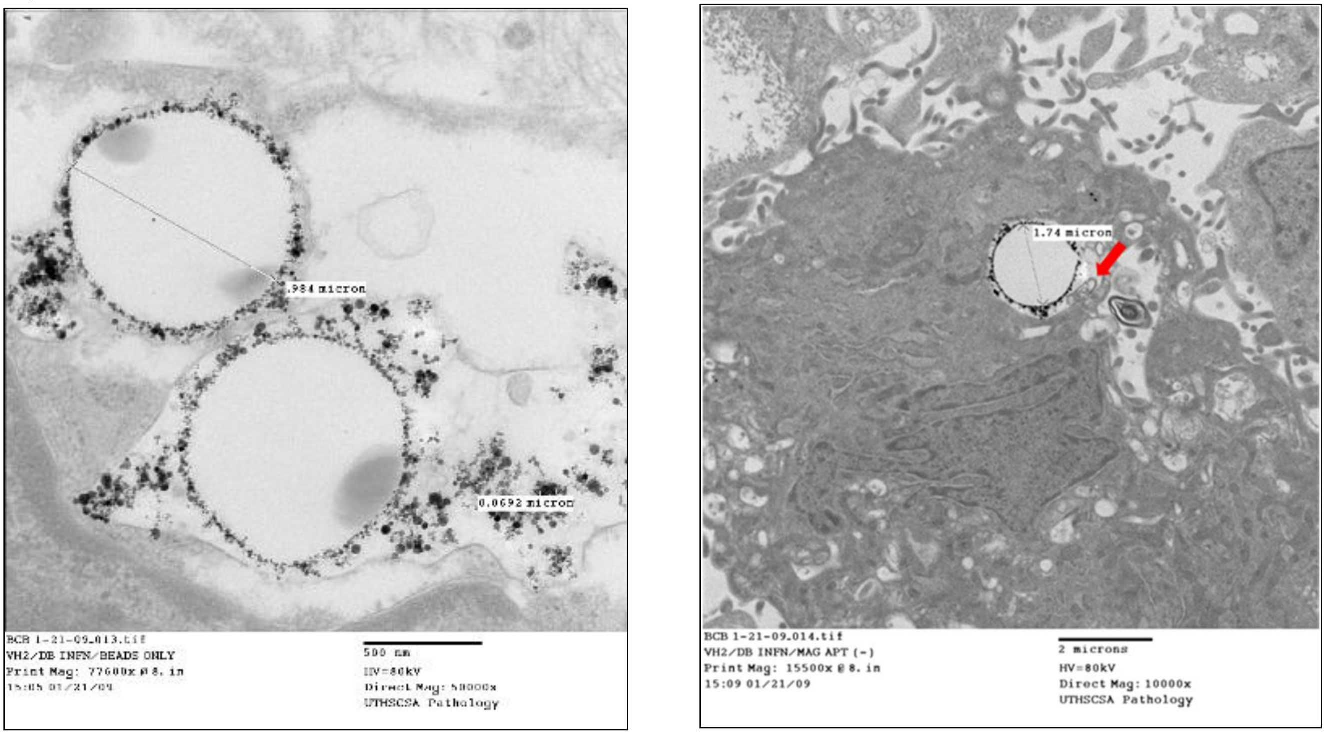


Figure 12a, 12b. Transmission Electron Microscopy (TEM) images providing examples of the polystyrene 1.17µm bead being engulfed. 12B, presence of bacillary body highlighted with arrow adjacent to bead. On higher magnification, bridge connecting the two can be seen.

11 Molecular Analysis

11.1 Overview

The decision was made to place a major emphasis on attaining reliable molecular data on the Viper Plague bacterial and viral components with the goal of identifying both organisms through molecular genotyping methods. An investigation was conducted into the work and current protocols used to achieve this goal. The results of the investigation revealed a possible need to adapt the classical molecular techniques used to better suit the identification of the Viper Plague constituents and to streamline the process. It was also realized that extensive troubleshooting would possibly be needed at every phase of this process. For the microbial identification of the Viper Plague rickettsial bacterium, it was determined that the maintained bacterial organism originating from the Western Diamond-backed rattlesnake would be used because of the presence of only the rickettsial agent and that the classical method of sequencing the bacterial 16S rRNA (rrs) gene was the best approach. That entailed establishing a positive control of *Bartonella henselae* and keying on the most difficult aspects of identifying the rickettsial organism: the isolation of the intracellular pathogen from its host cell and the subsequent DNA extraction from the bacterium itself. Once these most difficult aspects had been successfully overcome, the bacterial 16S DNA fragment was sequenced to give a result which was partially verified by a

separate sequencing company. Additionally, other PCR assays were performed targeting certain genes specific to rickettsial organisms as another means to molecularly characterize the Viper Plague rickettsial agent. Identification of the retroviral organism was attempted, but was not completed.

11.2 Molecular Identification of the Rickettsial Organism

11.2.1 Bartonella henselae

Bartonella henselae, the etiological agent of Cat Scratch Fever, is a facultative intracellular bacterium phylogenetically grouped with the rickettsiae in the Order Rickettsiales. This pathogen was included in the process of identifying the Viper Plague for the ultimate purpose of serving as the positive control for certain PCR assays. Also, working with *B. henselae* would alternatively serve to resolve the protocols that would be used before implementing them for the identification of the Viper Plague rickettsial organism. After fastidious growth of the organism had been established on 5% sheep blood agar, a DNA isolation was performed on the pelleted microbe using the Qiagen QIAamp DNA Minikit (catalog #51304), strictly following the company-provided protocol. Once the DNA from *B. henselae* had been attained, a PCR assay was performed attempting to amplify two separate DNA fragments from the bacterial 16S rRNA gene using previously reported primer sets (Weisburg et al, 1991) that were slightly modified; fD1_mod : rD1_mod and fD1_mod : rP2_mod. After some optimizing of the PCR assay, both amplicons were successfully amplified from the *B. henselae* DNA. From the following image (Figure 13), the higher molecular weight (larger) fragment, for primer set fD1_mod: rD1_mod, and the lower molecular weight (smaller) fragment, for primer set fD1_mod : rP2_mod, can be seen while the negative controls did not amplify product, validating the assay. The investigation then shifted to the microbial identification of the Viper Plague bacterium once *B. henselae* was established as the positive control.

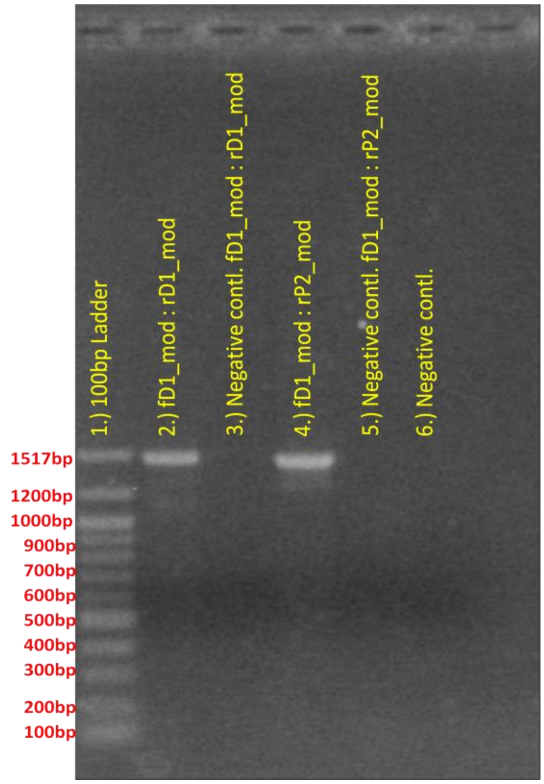


Figure 13. PCR assay amplifying bacterial 16S rRNA gene fragment from *Bartonella henselae* DNA, 1% TAE agarose gel.

11.2.2 Isolation of the Rickettsial Organism from Infected Host Cells

For the isolation of the rickettsial organism from host cells, the Diamond-backed isolate was chosen for inoculation of VH2 cells because of evidence, from TEM imaging, indicating that the rickettsial bacterium was the only infectious agent present in the infection line. The VH2 cells were chosen as the host cell line over VSW cells because the VSW cell line naturally harbors an endogenous retrovirus. Up to this point, the main obstacle for the identification of the bacterial component of Viper Plague had been the ability to reliably isolate a high enough organism load from the host cells for DNA isolation. To meet this dilemma, many factors were tested for the optimization of bacterial release from the host cell: freeze/thawing, sonication, chemical lysing using Trypsin, and mechanical lysing using needles with extremely small diameters. Also, it was understood from researching literature on rickettsial investigations that an increase in bacterial load was something that needed to be achieved; so, the Viper Plague rickettsial bacteria was amplified, in terms of total bio-mass, from six-well plates to multiple T150 flasks. The use of ribonucleotide inhibitors was also tested for the purpose of eliminating, or at least limiting, the amount of host cell ribonucleotides present in Viper Plague infected samples.

After testing the different factors in different combinations, a protocol was formulated that repeatedly yielded positive results. To isolate the intracellular bacteria from the host cell,

infected VH2 cells were chemically lysed with 1% Trypsin and mechanically lysed using 18ga and 27ga needles. The bacteria were then separated from the host cell debris using low-speed and then ultra-speed centrifugation. After pelleting the bacteria, sample preparations were treated with ribonucleotide inhibitors, DNase I and RNAse A, which were subsequently inactivated with 0.25M EDTA. Preparations were then cleansed with a series of washes with 1X PBS pH 7.2, resuspended in PBS, and stored at 4°C until the following DNA isolation. To decrease the probability of sample contamination from occurring, the uninfected VH2 sample, which served as the negative control, was processed separately beforehand. Also, all steps were performed under aseptic conditions within a Bio-Safety Level II hood.

11.2.3 DNA Isolation from the Rickettsial Organism

DNA extractions on the preparations of the isolated rickettsial organisms from host cells were performed using the Qiagen QIAamp DNA Minikit (catalog #51304) with slight modifications to the company-provided protocol to compensate for our samples having such a low amount of bacterial load. DNA isolation was performed under aseptic conditions within a Bio-Safety Level II hood. Samples were centrifuged at the beginning of the protocol at 30,000x*g* for 10 minutes instead of 5,000x*g* for 5 minutes, and then processed according to the Qiagen protocol. For the elution of the DNA samples at the end of the isolation, samples were eluted three times with 50µL of the Qiagen elution buffer instead of three times with 200µL. This modification allowed for a greater concentration of the target DNA. Uninfected VH2 samples, designated as the uninfected controls, were included and processed identically as the infected samples but at a different time beforehand. To accurately quantify the amount of DNA in each sample, it was discovered that the Nanodrop 1000 instrument worked best for attaining concentrations of samples. The isolated DNA samples were run on a 1% TAE gel to visualize the yield and purity of the DNA isolation process. Gel results were then captured by autoradiography to produce an electronic record of the gel image. The results from the following gel image (Figure 14) show that DNA was successfully extracted from our infected culture sample, a feat that had not been previously accomplished until now.

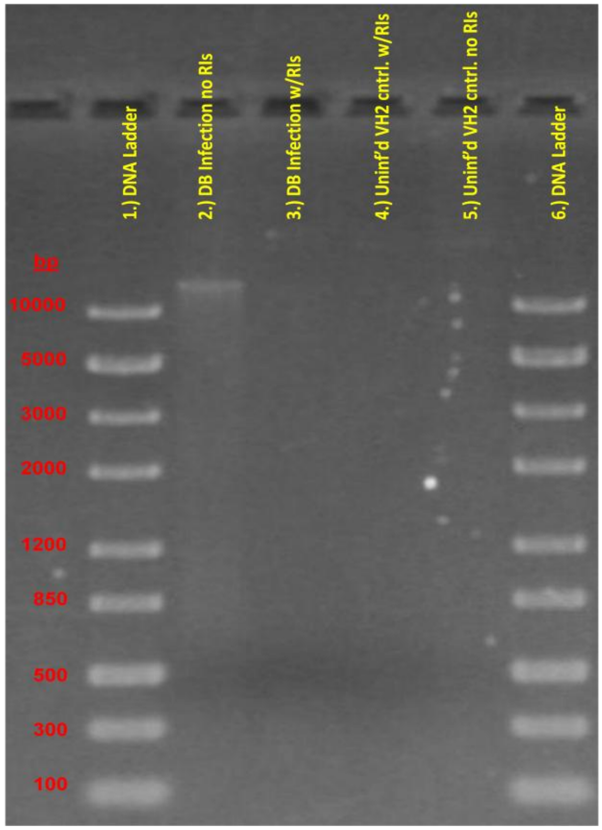


Figure 14. Gel check of the DNA isolation from the Viper Plague rickettsial organism sub-cultured from the Western Diamond-backed rattlesnake, 1% TAE agarose gel.

11.2.4 Bacterial 16S gene

After verification of the DNA preparations had been completed, PCR assays were performed attempting to amplify the two separate DNA fragments of the bacterial 16S rRNA gene from the isolated Viper Plague bacterium DNA with the aforementioned primers that were used to establish the Bartonella positive control, fD1_mod : rD1_mod and fD1_mod : rP2_mod. Early efforts to amplify the approximate 1500bp fragments for DNA sequencing proved unsuccessful and required much troubleshooting in order to optimize the PCR reaction. Once it had been proven repeatedly that both primer sets were successful in amplifying the region of interest, sequencing was pursued using only the primer set that produced the larger of the two fragments, fD1_mod: rD1_mod. Figure 15 shows the amplification of the bacterial 16S DNA fragment using the fD1_mod: rD1_mod primer set with the included controls:

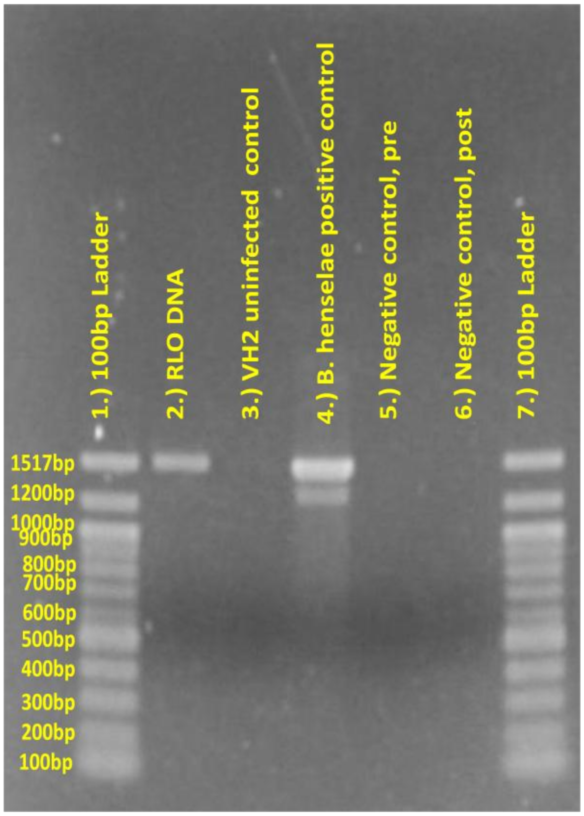


Figure 15. PCR assay amplifying the bacterial 16S rRNA gene fragment from the Viper Plague rickettsial DNA, from the Western Diamond-backed rattlesnake, 1% TAE agarose gel.

11.2.5 Rickettsial-specific 16S Fragment

Multiple PCR assays were performed on the isolated Viper Plague bacterium DNA attempting to amplify a portion of the bacterial 16S rRNA gene specific to the Rickettsia spp. by using a PCR assay referenced from Francisco J. Marquez et al., 1998. The investigating group identified a nucleotide sequence within the 16S gene and confirmed, through BLAST analysis, that the sequence was specific to Rickettsia spp. Used in conjunction with the fD1_mod forward primer from the general bacterial 16S assay, the assay was shown to reliably amplify a 520bp product. RHPC scientists wanted to replicate Marquez's Rickettsial-specific 16S assay in hopes of producing the 520bp amplicon which would be a positive indication that the bacterial agent of Viper Plague is definitely an organism from the genus Rickettsia. Unfortunately, the PCR assay failed to produce any type of amplification. Troubleshooting of the assay was performed by modifying the PCR program but to no avail.

11.2.6 pCS20 Gene

In trying to molecularly characterize the unknown Viper Plague rickettsial organism, the original idea that the bacterium may have been Ehrlichia ruminantium could now be tested by PCR analysis because of the availability of isolated DNA. *Ehrlichia ruminantium* is the biological agent that causes Cowdriosis (Heartwater), a highly infectious livestock disease with an extremely high mortality rate. To date, *E. ruminantium* or cases of Heartwater have not been identified on U.S. soil.

PCR assays that detect *E. ruminantium* infections were performed targeting the pCS20 genomic region of this intracellular pathogen. For one assay, two sets of primers referenced by Van Heerden et al., 2004 were used. The first primer set, pCS20F: pCS20R, produces a 531bp DNA fragment while the second, pCS20F: HpCS20R, produces a 1071bp DNA fragment. In another assay, a nested PCR was attempted using the primers AB128, AB129, and AB130 referenced from the paper by Martinez et al., 2004 All DNA samples for the rickettsial organism isolated from the Western Diamond-backed isolate failed to produce an amplified product in both assays. However, the results can be considered inconclusive because positive *E. ruminantium* DNA was not available therefore not included.

11.2.7 gltA Gene

The citrate synthase (gltA) gene is another molecular tool that is used by investigators for the microbial identification of rickettsial organisms. The gltA gene helps confirm infection and is commonly targeted because it has been detected in all rickettsial species. The primer set, CS-239: CS-1069, has been shown by Labruna et al., 2004 to amplify a 834bp region of the gltA gene that is specific to the Genus Rickettsia. A gltA PCR assay was performed on isolated bacterial DNA, from Viper Plague infected host cells from the DB infection line, using the CS-239: CS-1069 primer set to confirm the Viper Plague bacterial organism and for planned identification of it through genomic sequencing of the amplicon.

The following gel image (Figure 16) shows the results for the gltA PCR assay. A multiple banding pattern can be seen for the DNA which is a result of non-specific binding of the primers due to the PCR program's low annealing temperature. Lower annealing temperatures allow the primers to non-specifically anneal to different regions of the template DNA that are similar in homology to the target region. A multiple banding pattern can be also seen for the uninfected VH2 sample, an indication of some level of contamination for that sample which was not unexpected. This sample repeatedly presented amplification of the bacterial 16S amplicon for that particular assay. The *B. henselae* DNA sample also produced a multiple banding pattern, but significantly different from the unknown rickettsial agent and uninfected VH2 samples. *B. henselae* is grouped with the rickettsiae in the Order Rickettsiales and is known to also possess the gltA gene. Even though the primers for this assay share an approximate 40% homology to the genomic target regions within the *B. henselae* gltA gene, the low annealing temperature allowed the primers to bind then amplify the same 834bp amplicon. On closer observation,

this 834bp DNA fragment is present in only the Bartonella lane (lane 4) and not the lanes for the rickettsial organism (lane 2) or the uninfected VH2 cells (lane 3).

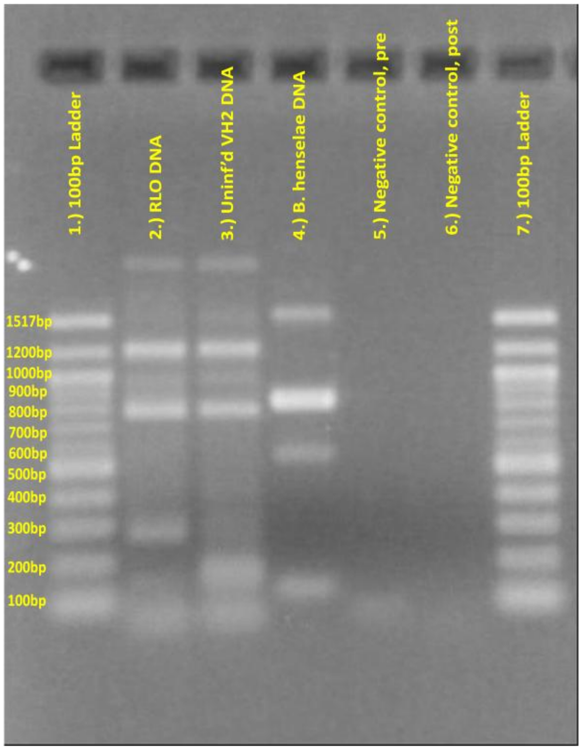


Figure 16. PCR assay amplifying the gltA gene fragment, 1% TAE agarose gel.

11.2.8 Preparation of Bacterial 16S Amplicon for Genomic Sequencing

For microbial identification of the Viper Plague bacteria found in the DBisolate, genomic sequencing of the bacterial 16S DNA fragment amplified from the isolated Viper Plague bacterial DNA was undertaken. Efforts to have the pathogens 16S DNA fragment sequenced were only commenced once the PCR assay had been shown to be consistently reproducible and that the whole process was considered to possess a high level of quality assurance. A scaled-up bacterial 16S PCR reaction, using primers fD1_mod and rD1_mod, was performed to increase the final concentration of the amplicon. The total PCR reaction volume was run on a 1.5% SeaPlaque agarose preparative gel and excised from the gel (Figure 17).

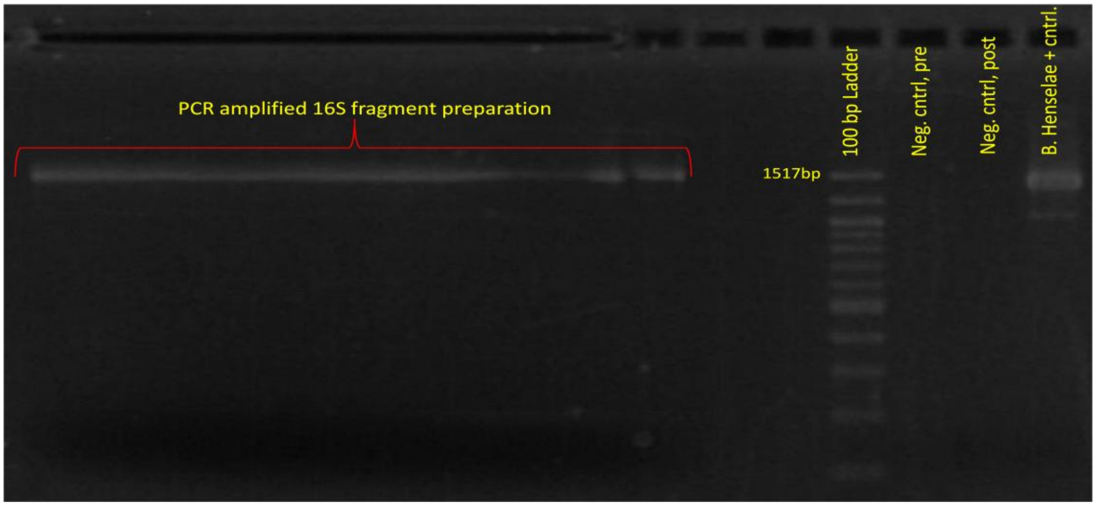


Figure 17. Preparative gel for PCR amplification of bacterial 16S rRNA fragment, 1.5% SeaPlaque agarose gel.

The 16S fragment was purified from the gel using the QIAquik Gel Extraction kit (catalog #28704). The purified DNA was visualized on a 1% TAE agarose gel to confirm the yield and purity of the DNA preparation (Figure 18). The 16S DNA amplicon was purified as two individual samples because the weight of the gel fragment was too large, surpassing the Qiagen kit's protocol restrictions.

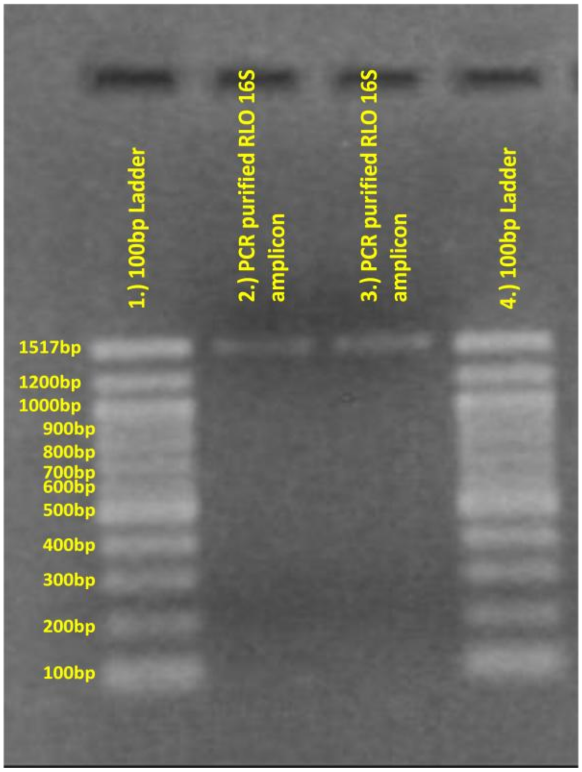


Figure 18. Validation of the purified 16S rRNA fragment from the suspected Viper Plague rickettsial organism, 1% TAE agarose gel.

A 16S amplicon sample, purified using Qlagen's PCR Purification kit was sent for sequencing at Segwright (Houston, Texas). Purified DNA samples utilizing the QIAQuik Gel extraction kit were submitted to the UTHSCSA's Advanced Nucleic Acid Core Facility for sequencing as well.

11.2.9 <u>Results of Genomic Sequencing</u>

Once the genomic sequences were received from UTHSCSA's Core Facility, a BLAST analysis was performed of the contig'd sequences for both samples. The results from the BLAST analysis convincingly show that the putative rickettsia-like organism contigs for both samples matched 100% to the 16S sequence from *Comamonas testeroni*. The staff from UTHSCSA's Core Facility were promptly contacted, and they confirmed that the submitted DNA samples appeared, by sequencing, to have been clean samples. Inquiries into whether a second DNA template may have been present in the samples returned a definite response that only one DNA template was present and that the sequencing of the samples was very straightforward. The chromatogram below (Figure 19) provides a section of the sequence data highlighting the purity of the reaction.

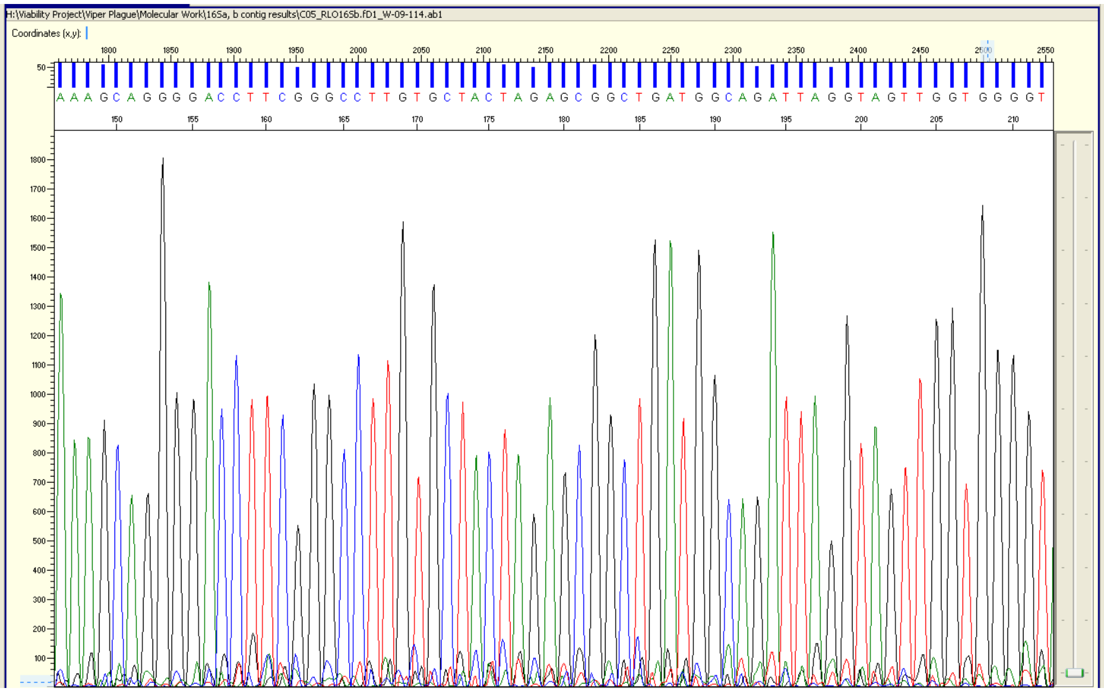


Figure 19. Chromatogram showing section of sequence data for rickettsial-like organism sample.

The results from the UTHSCSA Core facility were substantiated when the DNA sample submitted to Seqwright yielded a partial ~500bp sequence region matching Comamonas as well.

11.3 Molecular Identification of the Retrovirus of Viper Plague

Molecular analysis of the Viper Plague retrovirus was conducted by performing a PCR assay, using complimentary DNA (cDNA) reverse transcribed from the retrovirus, screening for the presence of the retroviral specific gag gene. The gag gene encodes for the Gag polyprotein which is essential for the assembly and release of retroviral particles from infected cells**.** The virions were isolated from cell culture supernatant using the Virabind Retrovirus Concentration and Purification kit (Cell Biolabs, Inc.; catalog #VPK-130). The RNA was isolated from the purified virion pellet by using Tri-Reagent (Molecular Research Center, Inc.; catalog #TR118) and following the accompanying product protocol. cDNA was reverse transcribed from the viral RNA by using M-MLV Reverse Transcriptase (RT) (Applied Biosystems, catalog #AM2043) and following the provided company protocol. Degenerate primers, gag1 and gag2 (Ristevski et al., 1999), were then used to perform a PCR reaction attempting to amplify either a 119bp or 157bp amplicon. PCR samples were visualized on a 1% TAE gel.

The RT-PCR did not produce any amplification of the cDNA. It was observed that the Nanodrop instrument was able to determine a concentration yield for the samples after the

RNA isolation and preparation of the cDNA, but neither could be visualized when a small volume of each was run on a test gel. This is an enigma, especially in light of previous success in producing electrophoretically identifiable PCR products from pol and gag primers (2).

12. Mycoplasma Testing

12.1 Background

Contamination of the infected and uninfected cell lines was suspected because of the aberrant changes observed in host cell morphology and the slight alterations in the infection sites. Also, the PCR amplification of uninfected VH2 controls further supported our suspicion when bands were observed repeatedly in the VH2 negative controls even though extra measures were taken to prevent cross-contamination of DNA samples at every phase. A Mycoplasma spp. bacterium was considered as the source of the contamination because of its reported high incidence rate of involvement in cases of lab contamination. The other alternative considered was that the VH2 cells had fulfilled their life expectancy. As a precaution, the VH2 cell line was re-started from freezer stock. Due to the possibility that Mycoplasma contamination may have been present in our maintained cell lines, commercially available Mycoplasma Detection kits were ordered to test that possibility. Because of the potential for contamination, testing for Mycoplasma would now be an established standard procedure in the identification of unknown intracellular pathogens.

12.2 Lookout PCR Mycoplasma Detection Kit

The Lookout Mycoplasma PCR Detection kit (Sigma-Aldrich, catalog #MP0035) was used to detect Mycoplasma contamination of infected and uninfected cell lines. Optimization of the Mycoplasma Detection assay had to be performed after the initial use of the kit showed no amplification of any test sample or control. The positive and negative controls were focused on, as a means to be certain the assay had performed appropriately. Figure 20 shows, after some troubleshooting, the correct amplicon sizes for both the negative control (481bp) and positive control (259bp). The positive control lane contains one extra band, but after speaking to a representative from Sigma, the presence of the extra band at that particular size is only an indication of the sensitivity of the detection kit.

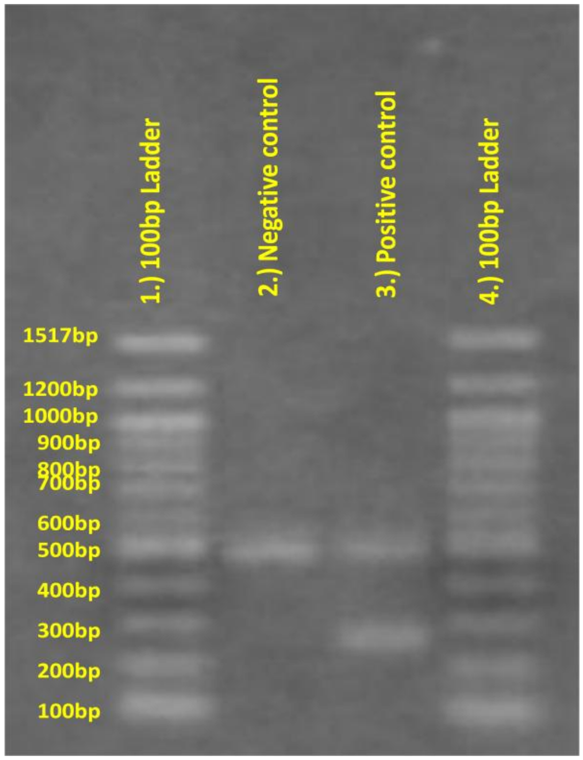


Figure 20. PCR optimization of the internal negative and positive controls for Lookout Mycoplasma PCR Detection kit, 1% TAE agarose gel.

Once the assay for the Lookout Mycoplasma PCR Detection kit had been optimized, samples were screened for Mycoplasma contamination. The uninfected cell lines that were currently being maintained were tested. The test samples included two samples of VH2 cells at different passage numbers and a sample from the HeLa cell line. The VH2 sample with the lower passage number and the HeLa sample produced bands at approximately 481bp which is equivalent to the negative control, an indication that these samples did not harbor Mycoplasma contamination (Figure 21). The positive control amplified the expected band at approximately 259bp plus the additional band equal to the negative control, which has been previously clarified as being acceptable. The VH2 sample with a higher passage number failed to produce an amplicon of any size. This may be explained by the fact that this sample had failed to be recently sub-cultured before testing leading to the production of PCR inhibitory factors, which interfered with the efficiency of the assay. This observance is consistent with the information given in the product manual.

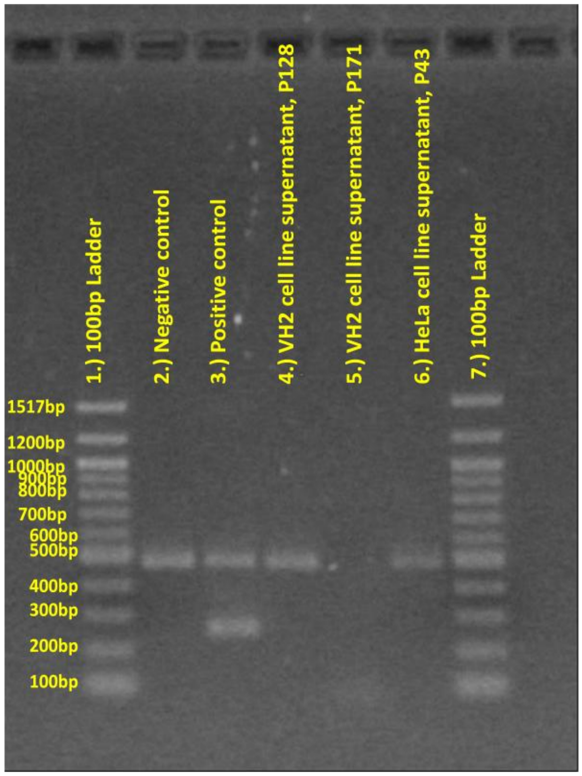


Figure 21. Mycoplasma contamination testing of lab samples using Lookout Mycoplasma PCR Detection kit, 1% TAE agarose gel.

12.3 <u>VenorGeM® Mycoplasma Detection</u>

The VenorGeM® Mycoplasma Detection kit (Minerva Biolabs, catalog #MP0025) was a more comprehensive Mycoplasma detection kit that was incorporated into the lab due to the fact that it detects all the Mycoplasma species detected by the Lookout kit plus Mycoplasma pnuemoniae, covering the full spectrum of all known Mycoplasma species. It also includes a boiling step allowing for a more efficient reaction to take place between the target DNA and the PCR reagents. For the first use of the kit, infected cell lines that were used to maintain the Viper Plague infectious organisms were tested along with uninfected HeLa cells. The PCR assay produced amplicons for all test samples equal in molecular size to the 191bp band for the internal negative control (Figure 22); therefore, indicating the samples were negative for Mycoplasma contamination. As a quality control check, the internal positive control did successfully amplify a product at 191bp, the correct molecular weight per the kit protocol.

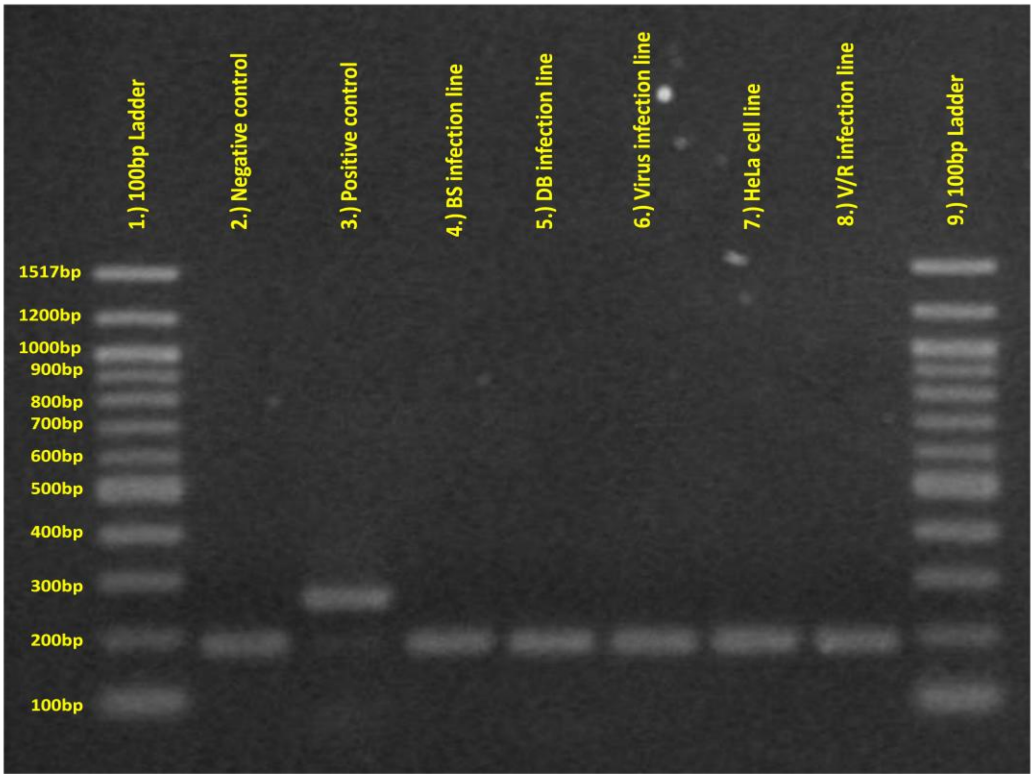


Figure 22. Mycoplasma contamination testing of lab samples using VenorGeM® Mycoplasma Detection kit, 1.5% TAE agarose gel.

As an extra measure to test for Mycoplasma detection, all DNA samples prepared up to this point were screened using the VenorGeM® Mycoplasma Detection. The PCR assay produced amplicons for all test samples equal in molecular size to the 191bp band for the internal negative control, denoting the samples as negative for Mycoplasma contamination. As a quality control check, the internal positive control did successfully amplify a product at the correct molecular weight. A faint band can be seen around the 191bp molecular weight for the internal positive control which is acceptable (Figure 23).

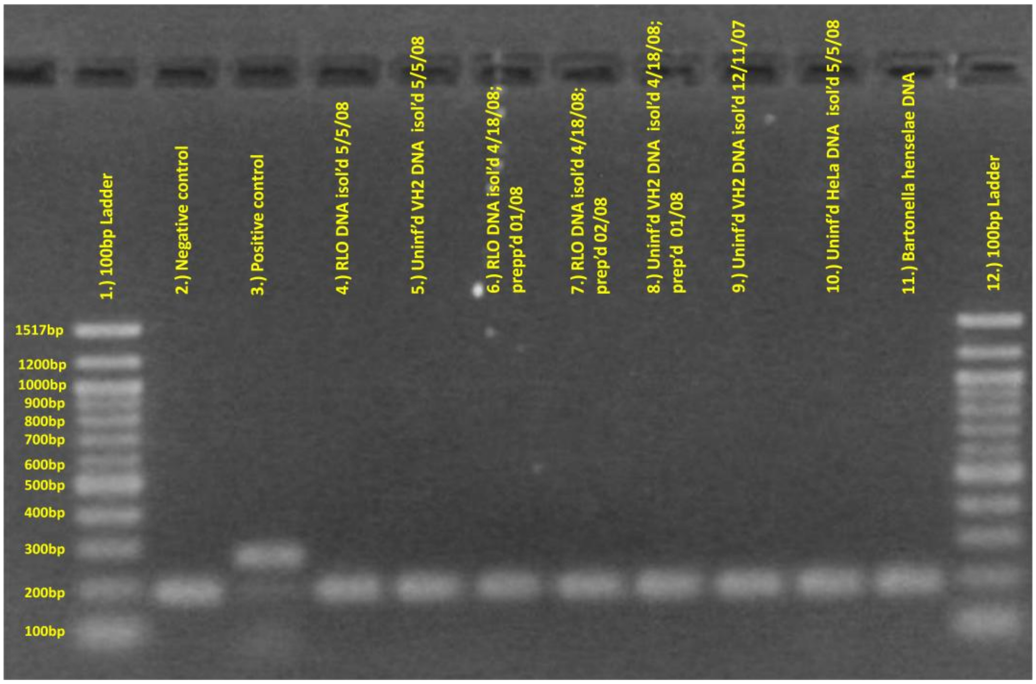


Figure 23. Mycoplasma contamination testing of DNA samples using VenorGeM® Mycoplasma Detection Kit, 1.5% TAE agarose gel.

12.4 Conclusion on Mycoplasma Contamination Testing

The Lookout Mycoplasma PCR Detection kit from Sigma-Aldrich and the VenorGeM® Mycoplasma Detection kit from Minerva Biolabs were incorporated into the lab because of the possibility that Mycoplasma detection may have been present in our samples. Mycoplasma bacteria are insidious microbes that are notorious for their high incidence rate of lab contamination. They are able to reside in cell lines, sometimes without ever presenting signs of their presence, and change not only the cellular morphology but also cellular processes. Once aberrant changes were seen in the maintained cell lines and the infection lines, the kits were utilized to gain some sort of confirmation. After extensive testing, it was decided that the VH2 cells were not contaminated by a Mycoplasma bacterium but, rather, were more than likely sub-cultured beyond their recommended passage limit. Also, it was confirmed that the HeLa cells, infection lines, and isolated DNA samples did not possess Mycoplasma contamination. The slight alteration in the infection sites was attributed to the state of the host cell. VH2 cells were fortunately already re-started from freezer stock by this point. As mentioned previously, testing for the contamination by Mycoplasma would be included as a standard procedure in the process for the microbial identification of unknown pathogens.

13. Supplemental Work

To further increase our knowledge of rickettsial pathogens, the 2007 and 2008 International Meetings on Rickettsiae and Rickettsial Diseases were attended. The purpose was to become familiar with the latest research on intracellular pathogens in the Order Rickettsiales and different rickettsial diseases. The conferences covered the current state of knowledge on the pathogenic agents that cause rickettsioses and provided the latest in methodologies utilized when working with these biological agents. In addition to learning a great deal of new and useful molecular techniques, important contacts were made with several experts in the field for possible future collaborations. A report summarizing all of the contacts made during the conference was prepared and submitted to the government customer.

14. Conclusion

From early research on the Viper Plague disease, which was isolated from a private herpetological collection, it was shown that the disease consisted of a rickettsial-like organism and a retrovirus. A new isolate from a Western Diamond-backed Rattlesnake (*Crotalus atrox*) was established in cell culture and was believed to be the Viper Plague microbe without the retrovirus. However, - abundant data has been gathered that strongly indicates that the rickettsial-like pathogen from the Diamond-backed rattlesnake is contaminated with DNA of or an actual bacterium from the Genus Comamonas. The latter is unlikely because bacterial cultures from the tissue cultures were sterile (Comamonas is a large non-fastidious soil and water microbe that should grow on non-selective medium) and no large filamentous bacilli were observed by light microscopy or electron microscopy in the tissue cultures. Furthermore, the light microscopy, electron microscopy, and OX19 aptamer and antibody studies yielded data compatible with the presence of a rickettsia rather than a free-living bacteria.

Even though the results from the attempted isolation of the rickettsia-like organism from the Diamond-backed rattlesnake samples were not expected, great strides were made in establishing a system of protocols for the identification of unknown pathogens, intracellular and extracellular. For rickettsia-like organisms, a conjugated fluorescent antibody and an iron nanoparticle conjugated aptamer against the OX19 common antigen of spotted fever and typhus class rickettsia were engineered that exhibited specific binding for the model target microbe that shares the OX19 antigen, *Proteus vulgaris*. . Furthermore, a novel magnetic collection method for intracellular rickettsial pathogens from field samples was designed based on the OX19/iron nanoparticle aptamer conjugate described above and holds a great deal of promise. However, before this collection method can be fully realized, with future research and development leading to a reliable detection/identification method for several kinds of pathogens, the DNA capture elements, aptamers, that are presently a part of this system will need to be tested for functionality.

References

1. Kiel, J. L., R. M. Alarcon, J. E. Parker, J. Vivekananda, Y. B. Gonzalez, L. J. V. Stribling, C. J. Andrews. 2006. Emerging tick-borne disease in African vipers caused by a *Cowdria*-like organism. Ann. N.Y. Acad. Sci. 1081:434–442.
2. Kiel, J.L., Gonzalez, Y., Parker, J.E., Andrews, C., Martinez, D., Vacheiry, N., LeFrancois, T. 2008.Viral association with the elusive rickettsia of viper plague from Ghana, West Africa. Annals of the New York Academy of Sciences 1149, 318-321.
3. Labruna, M. B., T. Whitworth, M. C. Horta, D. H. Bouyer, J. W. McBride, A. Pinter, V. Popov, S. M. Gennari, D. H. Walker. 2004. *Rickettsia* species infecting *Amblyomma cooperi* ticks from an area in the state of São Paulo, Brazil where Brazilian spotted fever is endemic. J. Clin. Micro. 42:90-98.
4. Márquez, F. J., M. A. Muniain, R. C. Soriguer, G. Izquierdo, J. Rodríguez-Baño, M. V. Borobio. 1998. Genotypic identification of an undescribed spotted fever group *Rickettsia* in *Ixodes ricinus* from Southwestern Spain. Am. J. Trop. Med. Hyg. 58:570-577.
5. Martinez, D., N. Vachiéry, F. Stachurski, Y. Kandassamy, M. Raliniaina, R. Aprelon, and A. Gueye. 2000. Nested PCR for detection and genotyping of *Ehrlichia ruminantium*. Ann. N.Y. Acad. Sci. 1026:106–113.
6. Ristevski, S., D. F. J. Purcell, J. Marshall, D. Campagna, S. Nouri, S. P. Fenton, D. A. McPhee, G. Kannourakis. 1999. Novel endogenous type D retroviral particles expressed at high levels in a SCID mouse thymic lymphoma. J. Virol. 73:4662-4669.
7. Van Heerden, H., H. C. Steyn, M. T. E. P. Allsopp, E. Zweygarth, A. I. Josemans, B. A. Allsopp. 2004. Characterization of the pCS20 region of different *Ehrlichia ruminantium* isolates. Vet. Micro. 101:279-291.
8. Weisburg, W. G., S. M. Barns, D. A. Pelletier, and D. J. Lane. 1991. 16S ribosomal DNA amplification for phylogenetic study. J. Bact. 173:697-703.